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Advances in Controlling Differentiation of Adult Stem Cells for Peripheral Nerve Regeneration

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Keywords

stem cell differentiation, nerve conduits, nerve regeneration, biomaterials, electrical cellular stimulation

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Advances in Controlling Differentiation of Adult Stem Cells for Peripheral Nerve Regeneration

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Adult stems cells, possessing the ability to grow, migrate, proliferate, and transdifferentiate into various specific phenotypes including, neuronal or glial cell types, constitute a great asset for biomedical applications involving peripheral nerve regeneration. Adult stem cell plasticity, in particular their ability to undergo transdifferentiation, is sensitive to various cell-to-cell interactions and external stimuli involving the interactions with physical, mechanical and chemical cues within their microenvironment. Various studies have employed different techniques for transdifferentiating adult stem cells from distinct sources into specific lineages (e.g., glial cells and neurons). These techniques include using chemical and/or electrical induction as well as cell-to-cell synergetic effects via co-culture along with the use of various 3D conduit/scaffold designs. Such scaffolds consist of unique natural and/or synthetic materials that possess controllable physical/mechanical properties that can mimic the natural extracellular matrix environment of the cells. However, the current limitations regarding the final fate of implanted transdifferentiated stem cell populations, non-scalable transdifferentiation protocols, and design of a conduit/scaffold that mimics the complex extracellular matrix microenvironment have required development of new strategies for the effective transdifferentiation of stem cells and their implantation. In this progress report, we present a comprehensive review of recent advances in the transdifferentiation of adult stem cells into particularly Schwann cells or neurons via different approaches (chemical and/or electrical stimuli or co-culture with different cells) along with multifunctional conduit/scaffolds materials and designs. We also included potential cellular mechanisms and signaling pathways associated with stem cell differentiation. We conclude the discussion with some of the challenges that still need to be overcome in the field and provide an outlook toward future research directions.

1. Introduction

Peripheral nerve (PN) injuries affect 2.8% of trauma patients, frequently leading to life-long disability, reduced quality of life, and heavy economic and social burdens.[1-3] The incidence of PN injuries is high world-wide and over 200,000 PN repair procedures are performed annually in the US alone, resulting in ~ $1.3 – 1.9 billion spent. [1-3] Current treatment strategies have shown limited success and often result in incomplete recovery with poor functional outcomes, especially for large PN injuries. Although the peripheral nervous system (PNS) has an intrinsic capacity to regenerate and regrow axons to a certain extent through the growth permissive Schwann cells (SCs), spontaneous nerve regeneration in PNS has resulted in poor functional outcomes.[3-5] In particular, the recovery from large PN gaps is difficult to achieve without any therapeutic intervention or cell-based therapy.[3]

The current gold standard for severe peripheral nerve transection injuries are autologous nerve grafts. However, this treatment strategy has significant disadvantages such as biological complexity, donor site morbidity, limited length of graft tissue availability, and the requirement of multiple surgeries.[1-3] As an alternative strategy, the application of cell-based nerve regeneration therapies holds considerable promise for treatment of large PN injuries. Schwann cells, which form the myelin sheath around peripheral axons, produce numerous factors that serve to promote regeneration including extracellular matrix (ECM) molecules and neurotrophic factors along with Schwann cell integrins. Among these neurotrophic/growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) enhance guidance and support for regenerating axons.[6-11] Hence, biodegradable nerve regeneration or guidance conduits (NGCs) bearing SCs have shown perhaps the most promising strategy for PN nerve repair. However, this strategy has not transitioned to clinical use because of limited availability, donor site morbidity, and slow *in vitro* growth of SCs.[12-14] As a new approach, *in*

vitro transdifferentiated somatic stem cells possessing SC-like or neural phenotypes have recently been explored for PN regeneration.[15-27] Adult stem cells, isolated and derived from bone-marrow, adipose tissue or other connective tissue sources, have considerable translational potential for cell-based nerve regeneration therapies using autologous transplantation due to the lack of ethical concerns, accessibility, plasticity, multipotent nature, and transdifferentiation ability into functional cell types. These stem cells also have the ability to repair tissue through paracrine activity by cytokine production; to release neurotrophic factors including, NGF, BDNF and GDNF.[15, 20, 21, 28, 29] However, this strategy has not widely transitioned into clinical use due to three main challenges: lack of reliability in controlling the final fate of the implanted cell population, especially over the regeneration period; non-scalable transdifferentiation protocols; and challenges in designing a multifunctional conduit that mimics the complex ECM microenvironment.[30, 31] Thus, there is a critical need to overcome these challenges to make stem cell-based strategies a viable solution for PN injury repair. This review article primarily focuses on recent advances regarding adult stem cell transdifferentiation strategies, along with various conduit designs and materials for stem cell-based therapies targeting PN injuries. Previous review manuscripts have focused primarily on biomaterials and conduit-based strategies [32] for peripheral nerve regeneration while others have included detailed information on stem cell types and sources and various clinical studies.[33, 34]. Here we focus on collating the latest research on adult stem cell differentiation strategies and mechanisms including those that involve chemical, electrical or mechanical cues as well as unique combinations of multiple cues. Moreover, this progress report reviews the current state-of-the art with regards to conduit/scaffold materials that facilitate PN regeneration via said cues.

2. Stem Cell Function, Differentiation and Fate Commitment

Somatic stem cells, obtained from various connective tissue sources, possess a number of qualities well-suited for cell-based therapies including ease of isolation, rapid *in vitro* growth,

efficient host tissue integration, and extended *in vivo* survival. These stem cells are also considered a promising cellular source to provide replacement of lost neurons and glial support cells for nerve regeneration.[35, 36] It is also possible to modify stem cells via stable transfection to enable expression of exogenous genes for an efficient therapy. Furthermore, *in vitro* and *in vivo* studies have demonstrated their transdifferentiation into SC-like phenotypes and/or neurons and integration into Bands of Büngner, facilitating axonal guidance and re-myelination through enhanced secretion of growth factors and ECM proteins upon *in vivo* transplantation.[37-39] The cell-to-cell contacts and paracrine signaling serves to modulate the active molecule-secreting capabilities of transdifferentiated stem cells, and also synergistically induces the secretory activity of endogenous SCs and macrophage accumulation near the site of injury.[39, 40]

Numerous growth factors (i.e. NGF, BDNF, GDNF, and neurotrophin-3 (NT-3)), along with ECM proteins (i.e. collagen I & IV, fibronectin and laminin) and neurite guidance proteins (i.e. netrin and ninjurin-2), produced by endogenous SCs and transplanted stem cells have a profound effect on nerve regeneration [39, 41-47]. Neurotrophic factors, including neurotrophin family members and CNTF, all act on the MAPK/ERK, PI-3K/Akt, and JAK/STAT3 pathways. These factors activate protein kinase A(PKA), Ras/phosphotidyl inositol 3'-phosphate-kinase (PI-3K)/Akt, and Ras/Raf/mitogen-activated protein kinase (MAPK) and MAPK/extracellular signal regulated kinase (ERK) signal transduction pathways, leading to their biological actions upon binding to their specific high-affinity cognate tyrosine kinase (Trk) receptor.[48]

In addition, it was further demonstrated that the macrophage accumulation in the injury area and immunomodulatory effects of stem cells, which can potentially reduce the adverse effect of inflammation and fibrosis following nerve repair, can also be maintained via the secretion of factors such as granulocyte and macrophage colony stimulating factor (G-CSF, M-CSF), interleukin-6, 7, 8, and 11 (IL-6, IL-7, IL-8, IL-11) and tumor necrosis factor-α (TNF-α).[45, 47] However, it was noted that some of the ECM proteins have inhibitory effects

on axonal regeneration which was shown to be circumvented through the degradation of these proteins via the secretion of matrix metalloproteinase-2 (MMP-2) to support regeneration.[49, 50] Each of these ECM proteins or growth factors secreted by the stem cells trigger different cellular pathways and therefore different mechanisms to mediate regeneration.[51] The type of stem cell, its source and differentiation status, along with the method of cell delivery and properties of scaffold material and design, determines the levels of ECM protein and growth factor secretion as well as the benefit for each mechanism.[37, 38, 47, 52]

Somatic stem cells can be directly transplanted without applying *in vitro* transdifferentiation procedures. In some studies, the positive effects of directly transplanting undifferentiated stem cells, either alone or in co-culture with Schwann cells, on neural regeneration have been reported, highlighting the disadvantages of *in vitro* transdifferentiation procedures such as extra time, effort and cost spent, with unnecessary delays and limiting clinical applicability[39, 43, 44, 53-64]. Moreover, this strategy was also used to provide in-situ and *in vivo* differentiation of transplanted stem cells in response to local stimuli around the transplantation site.[56, 63, 65-67] However, there are concerns regarding the potential of undifferentiated cells to differentiate into unwanted, non-neural lineages in the complex *in vivo* environment bearing various growth factors, ECM proteins and other dominant cells in the area. Hence, some studies reported undesired differentiation of undifferentiated stem cells in response to local stimuli *in vivo*, while others were not able to show this effect[68-70].

Stem cells can also be transplanted *in vivo* after *in vitro* transdifferentiation into SC-like phenotypes and/or neurons. The transdifferentiation of stem cells depends on multiple interacting factors in their microenvironment, including biological, chemical, physical, mechanical and structural cues which, in combination, result in complicated differentiation behavioral outcomes [71-78]. The *in vitro* chemical/biological stimuli-based transdifferentiation, involving the use of costly chemicals and growth factors, is the most commonly used

strategy. These transdifferentiation procedures involve exposure to combinations of chemicals and growth factors, such as, β-mercaptoethanol, all-trans retinoic acid, fetal bovine serum, forskolin, recombinant human bFGF, recombinant human platelet-derived growth factor-AA, and recombinant human heregulin β-1 depending on the specific cell lineage.[79-81] Although chemical induction-based transdifferentiation procedures work with high success rates, the cellular mechanisms and signaling pathways dominating the differentiation have not been elucidated in detail yet. A very recent study by Sharma et al.[51] conducted a detailed proteomic analysis upon MSCs transdifferentiation into SCs via chemical induction. Their results revealed that proteins involved in axonal guidance [82], vascular endothelial growth factor (VEGF) signaling [83], neuregulin and platelet-derived growth factor (PDGF) [84, 85] and IL-1, IL-8 and TNFR1 signaling [86, 87], were significantly regulated upon MSC transdifferentiation. Similarly, Jori et al.[88] investigated the molecular mechanisms behind the transdifferentiation of MSCs into neurons. Their findings suggested that the classical PKA pathway was activated through the guanine nucleotide exchange factor for the small GTPase Rap1 and Rap2 as a result of an increase in cAMP, which induced MSCs differentiation. The observed MEK–ERK signaling also contributed to neural commitment and differentiation of MSCs whereas CaM KII activity did not influence the neuronal differentiation significantly.

Considering the positive influence of electrical stimulation in neural and axonal regeneration, the effect of combined chemical and electrical stimuli or only electrical stimuli on somatic stem cell transdifferentiation has also been recently investigated.[89-95] The effect of various electrical parameters including, voltage, frequency and electrical field strength, on stem cell differentiation was investigated using various conductive scaffolds. However, there is no detailed mechanistic study describing the electrical stimuli based differentiation of stem cells in the literature. It was only hypothesized and reported that the promoted stem cell differentiation via electrical stimuli could result from the upregulation of specific signaling pathways including focal adhesion kinase (FAK) or mitogen-activated protein kinase

signaling (p38)[91, 96-100], MAPK, PI3K, ROCK[92, 101] and ERK pathway as well as the alteration of cellular membrane potential via hyperpolarization and/or depolarization, modification of ion channels, calcium channel activation[102-104] or the increase in intracellular reactive oxygen species (ROS) generation.[92, 105] For instance, Park et al.[92] proposed that electromagnetic fields (EMF) induce NADH oxidase activation that generates reactive oxygen species (ROS) at the plasma membrane. The increased ROS further phosphorylates EGFR which in turn leads to CREB activation through PI3K/Akt pathway. They hypothesized that the EMF-induced CREB phosphorylation can promote neuronal differentiation of BM-MSCs.

In addition to these different inductive stimuli, the significant effect of mechanical and structural properties of scaffolds on the transdifferentiation ability of the stem cells has also been investigated. [72, 77, 106-110] It was reported that matrix stiffness can regulate the transdifferentiation of MSCs into specific lineages, indicating that softer substrates promote neurogenic, adipogenic and chondrogenic fates, while stiffer substrates enhance myogenesis and osteogenesis.[72, 78, 110-115] Moreover, the culturing and transdifferentiation of stem cells on 3D scaffolds with controllable structural properties mimicking an ECM microenvironment has also shown to be a better approach than using traditional 2D tissue culture plates.[116] Although the effect of mechanical cues on differentiation is known, further investigation regarding the cellular mechanisms and related signaling pathway analysis is needed in order to understand the stem cell differentiation behavior. The mechanism of stiffness-mediated transdifferentiation of stem cells is considered to be associated with the communication between mechanical and biochemical signals which are based on integrins. The focal adhesions, such as focal adhesion kinase (FAK), are key proteins regulating the intracellular signaling mechanisms/pathways. Any potential mechanical stimulation causes conformational change of signaling molecules/proteins resulting in opening phosphorylation sites, kinase cascades activation, transport of intracellular signaling molecules and changes in gene expression.[116] For instance, Du et al.[117] reported that soft substrates modulated the

neurogenic differentiation of MSC by blocking the BMP/Smad signaling pathway through β1 integrin internalization. They observed an increase in the active form of β1 integrin in MSCs on soft substrates. The detachment of integrin-ECM protein complexes also increased leading to a decrease in the cell surface distribution of β1 integrin in MSCs. This situation brought about the activated integrin internalization through caveolae/raft dependent endocytosis, which further enhances the membrane localization of BMP receptor (BMPR). Following the endocytosis, the binding efficiency of BMPR to the ligands starts to decrease resulting in the inhibition of Smad 1/5/8 phosphorylation. This further leads to the expression of neuronal genes, including microtubule associated protein 2, neurofilament protein light chain and nestin.

In addition to the mentioned stimuli controlling the transdifferentiation, recently the positive effect of ultrasound on stem cell transdifferentiation was also reported. This study showed enhanced cell viability, proliferation and neural differentiation along with up-regulated gene and protein expressions in induced pluripotent stem cells–derived neural crest stem cells (iPSCs–NCSCs).[118]

The potential positive effects of transdifferentiation on *in vivo* viability, enhanced neurotrophic factor secretion, myelination, axonal growth and regeneration have been demonstrated.[18, 95, 119-123] However, it is difficult to maintain the *in vitro* differentiated state of the stem cells under dynamic *in vivo* conditions.

It was previously reported that *in vitro* transdifferentiated cells have a tendency to revert back to their original phenotype once *in vitro* transdifferentiation cues are removed under *in vivo* conditions.[124] Direct comparisons in the literature have portrayed either a small advantage for transdifferentiated cells [121, 125], a small advantage for undifferentiated cells[68, 126] or no significant differences[53, 57]. Although the necessity of transdifferentiation prior to transplantation has been hypothesized for the best outcomes, the fate commitment of transdifferentiated stem cells is still posing an issue to the clinical applicability of the direct transplantation of undifferentiated cells.[127] For instance, Walsh et al.[127] reported that *in vivo*

transplanted skin-derived precursor cells (SKPs) can differentiate into myelinating Schwann cells by responding to local cues and stay in the transplanted area for a minimum of 10 weeks. They noted that the fate of SKPs *in vivo* depends on the nerve environment and can be modified by inclusion of heregulin-1β.

In another approach, Shea et al.[128] observed that bone marrow-derived MSCs, transdifferentiated into SCs, were able to maintain their differentiated state in co-culture with neurons purified from embryonic dorsal root ganglia. They further proved that the cells continued to maintain their status even without exogenous transdifferentiation-inducing factors or neurons. On the other hand, Faroni et al.[129] observed that the withdrawal of transdifferentiation medium resulted in a rapid reversion from SCs back to stem cell-like characteristics for transdifferentiated adipose-derived stem cells. This was accompanied by significant reduction in gene and protein expression of growth factors for SC-like phenotypes. In contrast, Zhang et al.[130] injected Schwann-like cells generated via *in vitro* transdifferentiation of adipose-derived stem cells into a sciatic nerve lesion area after crush injury *in vivo*. They observed transdifferentiated cells throughout the sciatic nerve injury site up to 12 weeks after transplantation, while a small fraction of the cells differentiated into fibrocyte/fibroblast-like cells.

Considering these conflicting results present in the literature, efficient and scalable transdifferentiation procedures enabling successful differentiation and precise control of the final fate of the implanted cell population along with the design of multifunctional NGCs that mimic the complex ECM microenvironment are needed for desired stem cell-based neural regeneration therapy. A detailed summary of recent studies in the literature regarding transdifferentiation strategies and cellular mechanisms along with the use of nerve guidance conduit designs is presented in the following sections.

3. Nerve Guidance Conduit Designs and Materials

The transplantation or delivery of transdifferentiated or undifferentiated stem cells through nerve guidance conduits (NGCs) holds considerable potential for successful nerve regeneration. Proper material selection along with efficient implantable NGCs production methods and design strategy, involving various combinations of biological/chemical, physical and mechanical cues, should be considered to mimic the natural microenvironment of ECM for cell survival, growth, proliferation, and differentiation to achieve efficient nerve regeneration (Figure 1).[38, 52, 131-134]

The selected NGC material should be biocompatible and the degradation rate should be consistent with the nerve regeneration rate to allow sufficient support during the regeneration process.[52, 131, 132] The conduit microstructure should possess proper pore size, porosity and swelling ratio along with desired mechanical properties to allow efficient nutrient diffusion and limit scar tissue infiltration.[5, 135-137] In addition, this will allow available microenvironments supporting the cells capable of secreting neurotrophic factors at the site of regeneration.[122, 137] The NGC should be able to provide cues such as longitudinal micropatterns or growth factor gradients to provide directional guidance.[14, 19, 23, 137-141] It is also important for a NGC to be eligible for protein modification to increase cellular attachment or controlled/sustained growth factor release to mimic the ECM microenvironment.[137, 142-146] Another important consideration is the physical size of the conduit, which should have a sufficiently large internal diameter with proper wall thickness to provide available space for the regenerating nerve and for straightforward handling during surgery.[3, 147] In addition to these parameters, considering the inherent ability of neuronal cells to send electrical signals along axons, it is also important to have an electrically conductive NGC that is capable of propagating electrical signals to promote stem cell differentiation, function and neural regeneration.[148-150] The NGCs possessing the mentioned features can be produced through many well-defined techniques, including immersion precipitation particulate leaching[151], extrusion [152, 153], injection molding[154, 155], non-woven or woven mesh rolling[156, 157], centrifugal casting[158], spinning mandrel technology[159], film

casting plus rolling[14, 19, 137], spin casting plus dry-phase inversion[137], and molding plus freeze drying[122]. Some advanced fabrication techniques have also been developed for preparing scaffolds with more complex configurations, such as a multichannel NGCs[160, 161], or NGCs containing longitudinally aligned fibers[162], micro-grooves[40] or hydrogels[163] within their lumens.

A wide variety of biomaterials, mostly including natural (gelatin, collagen, chitosan, fibrin etc.), synthetic polymers (poly(lactide acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), polyhydroxybutyrate (PBH) etc.), carbon-based conductive materials and their unique combinations, have been tested to promote stem cell differentiation and functional recovery of injured nerves as discussed in detail in previous reviews.[32, 38, 131, 132] [3, 147]

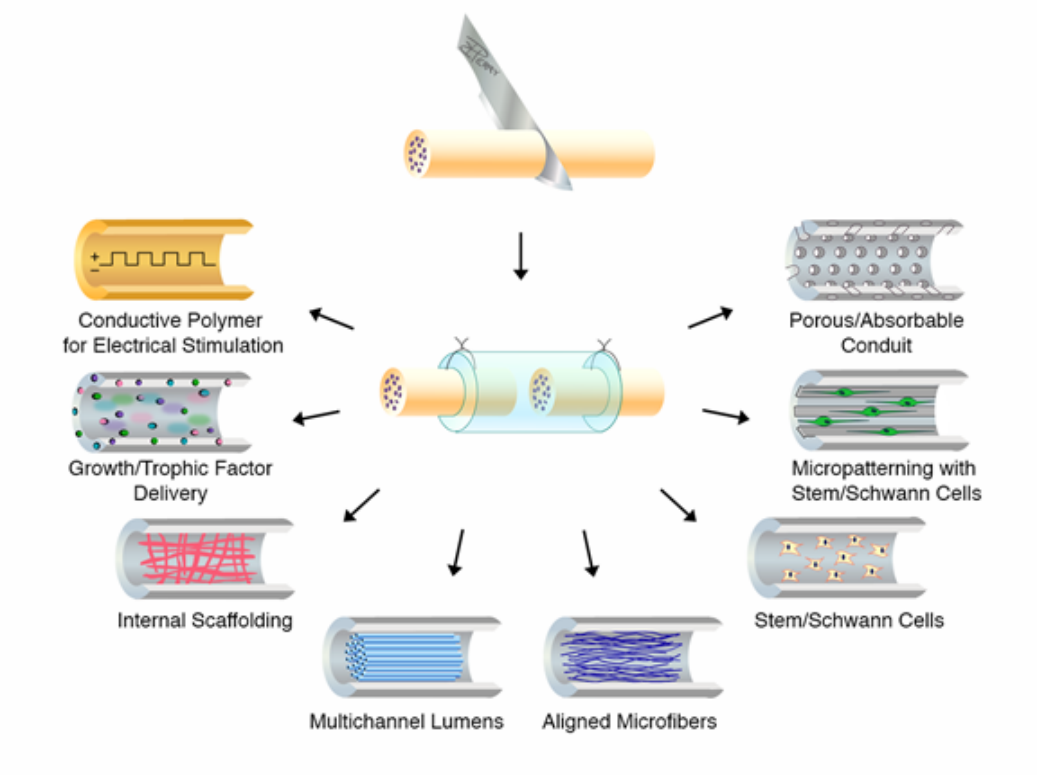

Figure 1. Conduit properties.[132] Illustration by Rose Perry. Copyright 2013, Woodhead Publishing.

4. Direct *In vivo* Stem Cell Transplantation and in situ Differentiation

The direct *in vivo* transplantation of undifferentiated stem cells *via* injection or *via* conduits made from various materials and possessing different features has been considered as a practical and clinically translational approach for PN injury repair. The transplanted stem cells have the potential to induce the production of in situ molecules and modulate the local environment for the reduction of inflammation and promotion of axonal regeneration through direct or indirect cross-talk with local glial cells. In addition, upon transplantation, these stem cells have a tendency to go through a process of transdifferentiation into different lineages as a result of synergetic interactions with their local environment. A summary of the studies using direct *in vivo* stem cell transplantation and in situ differentiation is summarized in Table 1.

Table 1. Summary of direct *in vivo* transplantation of stem cells and in situ differentiation.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
Acellular conduit made from a separated esophageal submucous membrane	Mouse skeletal muscle-derived multipotent stem cells (Sk-MSCs)	*In vivo* transplantation and in situ differentiation	In situ differentiation of Sk-MSCs into SCs and perineurial/endoneurial cells leading to enhanced axonal regeneration.	Tamaki et al.[66]
Acellular vein filled with a small piece of fresh muscle	Bone marrow-derived stromal cells (BMSCs)	*In vivo* transplantation and in situ differentiation	Enhanced regeneration accompanied by the in situ differentiation of BMSCs into SCs after the implantation.	Nijhuis et al.[56]
Autologous vein conduit filled with fibrin scaffold	Human adipose tissue-derived mesenchymal stem cells (hAdMSCs)	*In vivo* transplantation and in situ differentiation	Xenotransplantation of hAdMSCs into a fibrin scaffold promoted nerve regeneration in the peroneal nerve of rabbits, however no sign of differentiation was reported.	Lasso et al.[126]
Genipin-cross-linked gelatin conduit annexed with	Adipose tissue-derived stem	*In vivo* transplantation and in situ	Demonstrated in situ neuronal differentiation	Shen et al.[65]

tricalcium phosphate ceramic particles	cells (ADSCs)	differentiation	of ADSCs.	
Chitosan conduit with different degree of deacetylation and molecular weight	Bone marrow-derived mesenchymal stem cells (BMSCs)	*In vivo* transplantation and in situ differentiation	Chitosan conduit with 95% degree of deacetylation and molecular weight of 106 Da promoted the survival and outgrowth of cells, as well as differentiation of BMSCs into neural stem cells which induce bridging of 8-mm-long neural gap *in vivo*.	Zheng et al.[63]
Multi-channel chitosan conduits	BMSCs	*In vivo* transplantation and in situ differentiation	Enhanced nerve regeneration through the *in vivo* differentiation of BMSCs as efficient as nerve allografts in bridging the large gap in sciatic nerves of adult rats.	Zheng et al.[67]
Collagen based conduits	Mobilized dental pulp stem cells (MDPSCs)	*In vivo* transplantation and in situ differentiation	Implanted MDPSCs promoted axon regeneration through trophic activity acting on Schwann cells and promoting angiogenesis. No direct differentiation into SCs reported.	Yamamoto et al.[164]
Gelatin hydrogel tubes	ADSCs	*In vivo* transplantation and in situ differentiation	ADSCs transplantation promoted regeneration of axons, formation of myelin, and restoration of denervation muscle atrophy to levels comparable to those achieved by SCs transplantation. Cells survived for at least 4 weeks after transplantation without differentiating into SCs.	Sowa et al.[58]
Fibrin glue conduit	BMSCs	*In vivo* transplantation and in situ	Implanted MSCs enhanced axonal regeneration only when cyclosporine A	McGrath et al.[165]

		differentiation	treatment was applied. No differentiation observed.	
Poly-3-hydroxybutyrate (PHB) sheets	ADSCs	*In vivo* transplantation and in situ differentiation	No in situ differentiation of ADSCs was noted; however, the regenerative effect was attributed to released growth factors and/or indirect effect on endogenous SCs activity.	Erba et al.[53]
Polycaprolactone (PCL) based conduits	BMSCs	*In vivo* transplantation and in situ differentiation	Significantly improved the median nerve regeneration after a traumatic lesion suggesting that transplanted cells may occasionally transdifferentiate into SCs.	Oliveira et al.[166]
Poly (DL-lactide-e-caprolactone) copolyester based commercial conduit (Vivosorb, PLC)	Wharton's jelly derived mesenchymal stem cells (WJ-MSCs)	*In vivo* transplantation and in situ differentiation	No differentiation of WJ-MSCs into nerve structure was observed; however, enhanced regeneration accompanied by upregulation of expression for netrin-1, ninjurin, BDNF, GDNF, VEGF and angiopoitin-1 rat genes was reported.	Shalaby et al.[167]
Micropatterned poly(L-lactic acid) (PLA) nerve conduit	Neural stem cells (NSCs)	*In vivo* transplantation and in situ differentiation	Increased levels of IL12p80 triggering NSCs to SCs differentiation through Stat3 phosphorylation and enhanced functional and motor recovery.	Lee et al.[168]
Microstructured poly-caprolactone (PCL) filaments	BMSCs	Co-culture with SCs followed by *in vivo* transplantation and in situ differentiation	Demonstrated good axonal regeneration, myelination and reinnervation; however, no differentiation of MSCs into glial lineage.	Carrier-Ruiz et al.[169]

Local[170-172] and systemic[39, 171] administration of stem cells from different sources have been applied in order to enhance PN regeneration. It was reported that local injection exhibited significant increase in axonal fiber counts while systemic treatment resulted in improved nerve regeneration and pronounced electromotor recovery.[171] Although the injected stem cells (regardless of the injection method) showed immune modulatory effects, synergistically supported local Schwann cells, and enhanced PN regeneration with functional recovery through secretion of neuroprotective factors, their differentiation behavior was not consistent. Some studies reported efficient in situ differentiation of injected stem cells into various lineages[172] while the others did not observe any differentiation.[39, 170] Therefore, there is still uncertainty about the differentiation potential into specific lineages and maintaining the differentiated states long-term. Nevertheless, there is a therapeutic potential of the injected stem cells.

As an alternative to direct injection, the implantation of stem cells along with NGCs promotes the regeneration of damaged nerves by supporting and guiding axonal growth between nerve stumps and retaining neurotrophic factors, while preventing fibrous tissue ingrowth. In addition, the implanted stem cells can potentially differentiate into Schwann cells and provide a microenvironment enriched with growth factors, promoting nerve regeneration. Considering this, recent studies have used acellular esophageal submucous membrane[66] or vein conduits filled with fresh muscle[56] or fibrin scaffolds[126] bearing stem cells from various sources for cell implantation (see Table 1). Most of these studies demonstrated enhanced axonal regeneration using acellular conduits with stem cells; though only some studies observed in situ differentiation.[56, 66] Although acellular conduits are promising candidates to mimic an ECM microenvironment and support nerve regeneration and cell differentiation, their limited availability is still a challenge to their wide scale implementation.

Alternatively, bioengineered conduits with various properties made from natural biopolymers, synthetic polymers or combinations of the two were also used for direct implantation of undifferentiated stem cells. Chitosan conduits, with 95% degree of

deacetylation, possessing multi-channels were reported to promote the survival and outgrowth of cells, enhanced nerve regeneration as well as *in vivo* differentiation of BMSCs into neural stem cells.[63, 67] Chitosan was also used in combination with other biopolymers (chitosan/ silk fibroin scaffolds[70] or synthetic polymers (chitosan/PLGA[68, 69]) for different conduit designs providing efficient regeneration as in autografts. However, none of these studies reported in situ differentiation of stem cells. Similarly, other studies used different sources of stem cells with various conduits made from natural biopolymers (i.e. collagen based conduits[164], gelatin hydrogel tubes[58], fibrin glue conduit[165]) for *in vivo* implantation targeting nerve regeneration. All of these studies reported the localization of undifferentiated stem cells near SCs induced with several neurotrophic factors and the subsequent proliferation and migration of resident SCs that resulted in enhanced regeneration of axons, myelinated fibers, and revascularization. Some studies claimed that transplanted stem cells enhance homing and migration of endogenous cells to the injured site for remyelination through the stromal cell-derived factor (SDF-1)/CXCR4 signaling as reported in injured central nervous system.[164] Another study hypothesized that cyclosporine A, an immunosuppressive agent with known neuroprotective potential, promotes neuroregeneration and neuronal extension via induction of GAP-43 through the inhibition of romatase and calcineurin activity when stem cells implanted without immunosuppresion.[165]

However, very few studies observed the differentiation of implanted stem cells into different lineages. For instance, Shen et al.[65] demonstrated in situ neuronal differentiation of adipose tissue-derived stem cells (ADSCs) when transplanted in genipin-cross-linked gelatin conduits annexed with tricalcium phosphate ceramic particles. However, they did not conduct any study to elucidate the cellular mechanism of in situ differentiation.

The use of conduits made from different synthetic polymers, either alone or in combination (i.e. (Poly-3-hydroxybutyrate (PHB)[53], microstructured poly-caprolactone (PCL)[169], poly (DL-lactide-e-caprolactone) copolyester based commercial conduit (Vivosorb, PLC)[167]) with stem cells also promoted nerve regeneration, myelination and reinnervation

through released growth factors, indirect effect on endogenous SCs activity or upregulation of expression for certain genes or pathways such as netrin-1, ninjurin, BDNF, GDNF, VEGF and angiopoitin-1. However, they did not enhance the in situ differentiation potential of the transplanted stem cells. On the other hand, some studies reported occasional transdifferentiation of MSCs and NSCs into Schwann cells on implanted polycaprolactone (PCL)[166] and micropatterned poly(L-lactic acid) (PLA) based nerve conduits[168] (Figure 2), respectively. Based on the previous reports indicating the effect of Stat3 phosphorylation on astroglial differentiation of NSCs[173], Lee et al.[168] hypothesized and showed that IL12p80 could trigger differentiation of NSCs into Schwann cells through Stat3 activation, where IL12p40 subunit can bind to IL12 receptor β1 and then induce Stat3 phosphorylation and the downstream signaling pathway.

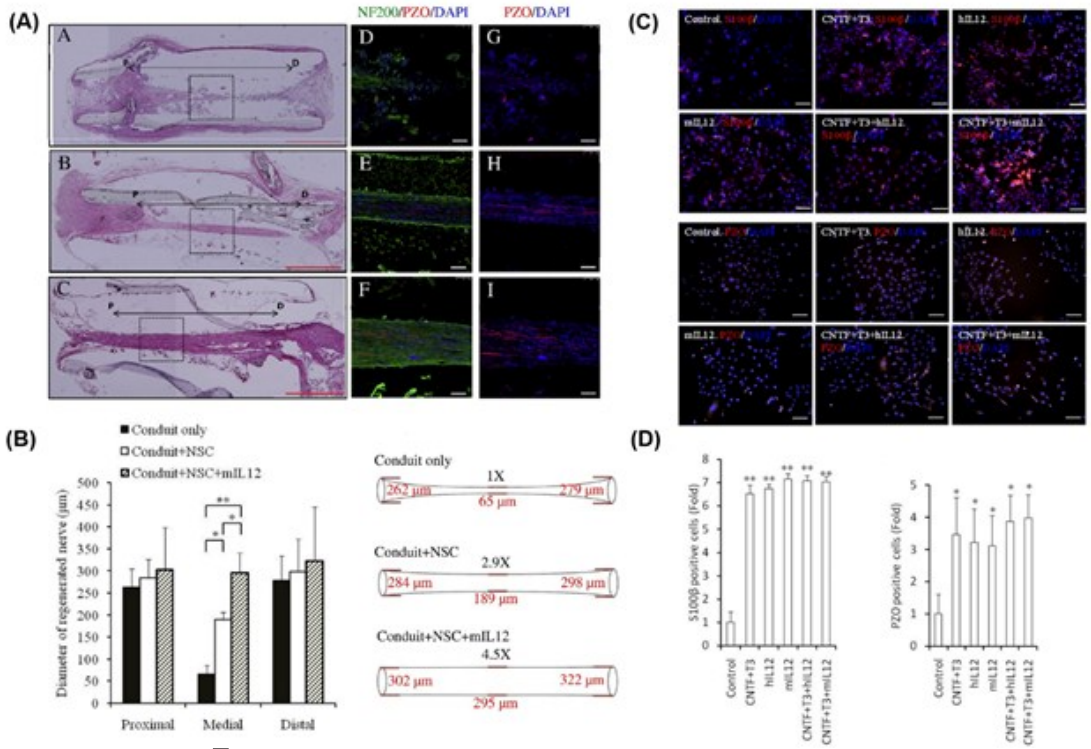

Figure 2. (A) Immunohistochemistry of regenerated sciatic nerve sections. The Conduit only group "A" showed less integration of regenerated axon than the Conduit + NSC group "B" and Conduit + NSC + mIL12 group "C". The failure of axon regeneration in the Conduit only group "D" was observed whereas myelinating Schwann cells (PZO positive cell) coupling nerve fiber (NF200 positive cell) existed in the medial region of conduits in the Conduit +

NSC “E” and in the Conduit + NSC + mIL12 groups “F”. **(B)** The schematic diagram of regeneration and quantitative results of regenerated nerve diameter at different sites. The regenerated nerve diameter was the highest in Conduit + NSC + mIL12 groups in the medial site. **(C)** Immunocytochemistry results. **(D)** Quantification of marker-positive cells. Immunocytochemistry staining and quantification results showed that IL12p80 could replace CNTF+T3 to induce Schwann cell differentiation of mouse NSCs. Reproduced with permission.[168] Copyright 2017, Elsevier.

5. Stem Cell Differentiation Using Chemical Induction

On 2D-Tissue Culture Plates

The growth of permissive Schwann cells (SCs), which are myelinating cells of PNS, form a myelin sheath around peripheral axons and produce extracellular matrix (ECM) molecules, integrins and neurotrophic factors, enhance guidance and provide support for regenerating axons.[6-9, 11, 174] The chemical stimuli-based transdifferentiation of stem cells into SC-like phenotypes was first conducted on regular 2D tissue culture plates as summarized in Table 2.

Table 2. Summary of direct transdifferentiation using chemical stimuli or conditioned media approach.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
2D tissue culture plates	Adipose tissue-derived stem cells (ADSCs)	Regularly used chemical induction based transdifferentiation	Transdifferentiation into SCs with ~95%. Demonstrated that SCs-like cells derived from ADSCs can undergo mitotic proliferation.	Fu et al.[175]
2D tissue culture plates	ADSCs	Olfactory ensheanthing cell conditioned medium based	Successfully differentiate ADSCs into SCs.	Xie et al.[176]

		transdifferentiation		
2D tissue culture plates	Neural stem cells (NSCs)	DMEM/F12 supplemented with forskolin, heregulin, bFGF, PDGF-AA and retinoic acid (RA)	Reported successful differentiation of NSCs into SCs exhibiting a spindle-like morphology and expressing the glial markers p75 and S100. Differentiated NSCs with enhanced neurite outgrowth of co-cultured NG108-15 cells.	Tong et al.[177]
2D tissue culture plates	Bone marrow-derived mesenchymal stem cells (BMSCs)	Lesioned microenvironment of the injured nerve by obtaining different extracts from the distal segment (Ds) and proximal segment (Ps) of degenerated rat sciatic nerves	Cells cultured with Ds extracts had significantly higher neural marker expression than that of cells cultured with Ps extracts and the untreated cells. The results showed SC-like differentiation.	Wang et al.[178]
2D tissue culture plates	ADSCs	Induction medium obtained by soaking cut rat sciatic nerve in culture medium for two days to extract the factors and agents	Observed a spindle shape like morphology and expression of the typical SCs markers upon induction proving successful differentiation.	Liu et al.[179]
2D tissue culture plates	Muscle-derived stem cells (MDSCs)	SC-conditioned medium	Differentiation of stem cells can be either manipulated using SC-conditioned medium or combined treatment of neurotrophic factors (PDGF, NT-3 and IGF-2) identified in the SC-conditioned medium giving the similar results.	Tang et al.[180]
2D tissue culture plates	ADSCs	Dissociation of nestin-positive non-adherent neurosphere cellular aggregates	SC-like differentiation providing expression of the characteristic SCs markers S100, p75 and GFAP and	Radtke et al.[181]

		produced from ADSCs cultured with bFGF and EGF and removal of mitogens	stimulated neurite outgrowth when co-cultured with dorsal root ganglion neurons.	
2D tissue culture plates	Tonsil-derived mesenchymal stem cells (T-MSCs)	Inducing T-MSCs to form neurospheres and incubating them with bFGF, EGF, and B27 supplement	Differentiation into SC-like cells and promoted myelination and regeneration of damaged nerves.	Jung et al.[182]
2D tissue culture plates	BMSCs	Infection of rat BMSCs by recombinant lentiviruses to obtain tropomyosin receptor kinase A (TrkA)-overexpressing BMSCs followed by direct transplantion using acellular grafts into rat sciatic nerve defects	Reported high degree of differentiation into SCs with the highest cellular survival compared to all other control groups	Zheng et al.[183]
2D tissue culture plates	Human pluripotent stem cells (hPSCs)	Combined sequential treatment with inhibitors of the TGF-β and GSK-3 signaling pathways, with neuregulin-1	hPSCs differentiated into immature SCs that were functionally confirmed by their secretion of neurotrophic factors and their myelination capacity *in vitro* and *in vivo.*	Kim et al.[184]
2D tissue culture plates	BMSCs	Chemical stimulation based on epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), neurobasal medium supplemented with N2 and B27, retinoic acid and sonic hedgehog	Reported successful NC-like differentiation and concluded that differentited cells could protect the disintegration and destruction of the injured peripheral nerve.	Guo et al.[24]
2D tissue culture	Human adipose derived stem	Inhibited the activin/nodal/TGF-β	Reported formation of neurite extensions,	Madhu et

plates	cells (hADSCs)	and BMP pathways using SB431542 and dorsomorphin, respectively	protein expression of neuron-specific gamma enolase, and mRNA expression of neuron-specific transcription factors and matured neuronal marker.	al.[26]

Dezawa et al.[16] were one of the first demonstrating the differentiation of bone marrow derived MSCs into SCs using a treatment based on exposure to beta-mercaptoethanol followed by retinoic acid and cell culturing in the presence of forskolin, basic-FGF, PDGF and heregulin. Following this study, other groups used similar protocols with minor modifications to achieve differentiation of adult stem cells from different sources (mostly bone marrow[20, 21, 178, 183] or adipose [46, 119, 175, 176, 179, 181, 185], human umbilical cord[186], muscle-derived stem cells (MDSCs)[180], tonsil-derived mesenchymal stem cells (T-MSCs)[182], human pluripotent stem cells (hPSCs)[184] and neural stem cells (NSCs)[177]) into SC-like phenotypes (see Table 2). For instance, in recent studies, differentiation of adipose derived stem cells (ADSCs) into SCs was obtained either using classical protocols mentioned above[175] or using olfactory ensheathing cell conditioned medium.[176] It was hypothesized that activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway and/or inhibition of the caspase pathway regulates MSCs to SC differentiation.[16, 175, 177] These studies also briefly described the roles of each cytokine used to differentiate stem cells into Schwann-like cells. β-ME increases the glutathione synthesis and reduces the cell response to oxygen tension. ATRT regulates the expression of cell surface receptors, retinoic acid receptors, retinoid X receptors and certain factors critical to the development of neural cell differentiation. HRG, a subtype of neuregulin, prevent apoptosis of SC precursors, induces neural crest differentiation into SCs selectively and acts on axonal signaling and myelination. FSK elevates the level of

intracellular cAMP which can mimic SC responses in the presence of axons during myelination *in vivo*. bFGF regulates MSC differentiation into the SC phenotype by involving in cell growth and differentiation.

Xie et al.[176] stated that secretion of growth factors and cell adhesion molecules constitutes the underlying mechanism of stem cell differentiation. Considering that SB431542, a small molecule inhibitor of the TGF-β/activin/Nodal pathway, is actively involved in cytokine- mediated cell proliferation and differentiation, they reported that blockade of TGF-β signaling can trigger differentiation of stem cells into neural cells.

Although multipotent NSCs have the potential to differentiate into various cells of neural lineage (neurons, astrocytes and oligodendrocytes) their differentiation into SCs has been rarely investigated. In the study of Tong et al.[177] the differentiation of NSCs into SCs was demonstrated using a slightly modified method of Dezawa et al.[16]. In general, these studies observed significant morphological changes, immunolabeling of various SC markers (i.e., p75, S100, GFAP, O4, etc.), regulated genes and proteins, and enhanced paracrine activity upon differentiation, thus supporting successful transdifferentiation. The biological activity of the secreted neurotrophic factors, such as NGF, GDNF, BDNF etc., was confirmed using a non-contact cell co-culture model with differentiated stem cells, DRGs or PC12 cells that can show neurite extension upon contact with the neurotrophic factors secreted from transdifferentiated MSCs. However, other studies have used different approaches to ensure differentiation of stem cells into SC-like phenotypes.

Based on the previously reported results indicating the potential for *in vivo* differentiation of transplanted stem cells into functional Schwann-like cells, some studies tried to mimic the lesioned microenvironment of the injured nerve by obtaining an induction medium through the extraction of factors and agents from the distal and proximal segment of degenerating rat sciatic nerves[178] or from the cut rat sciatic nerve soaked in culture medium[179] or directly using SC-conditioned medium.[180] These studies suggested that specific trophic factors secreted from sciatic nerve leachate or from the microenvironment of

the nerve lesions, were capable of inducing MSCs or ADSCs to differentiate into functional Schwann-like cells, which may potentially originate from the distal segment of the degenerated nerve, as in the case of regularly applied transdifferentiation protocol of combined neurotrophic factors (Figure 3).[178, 180] However, they stated that the mechanisms by which MSCs differentiate into Schwann cells *in vivo* and their functions are still uncertain.

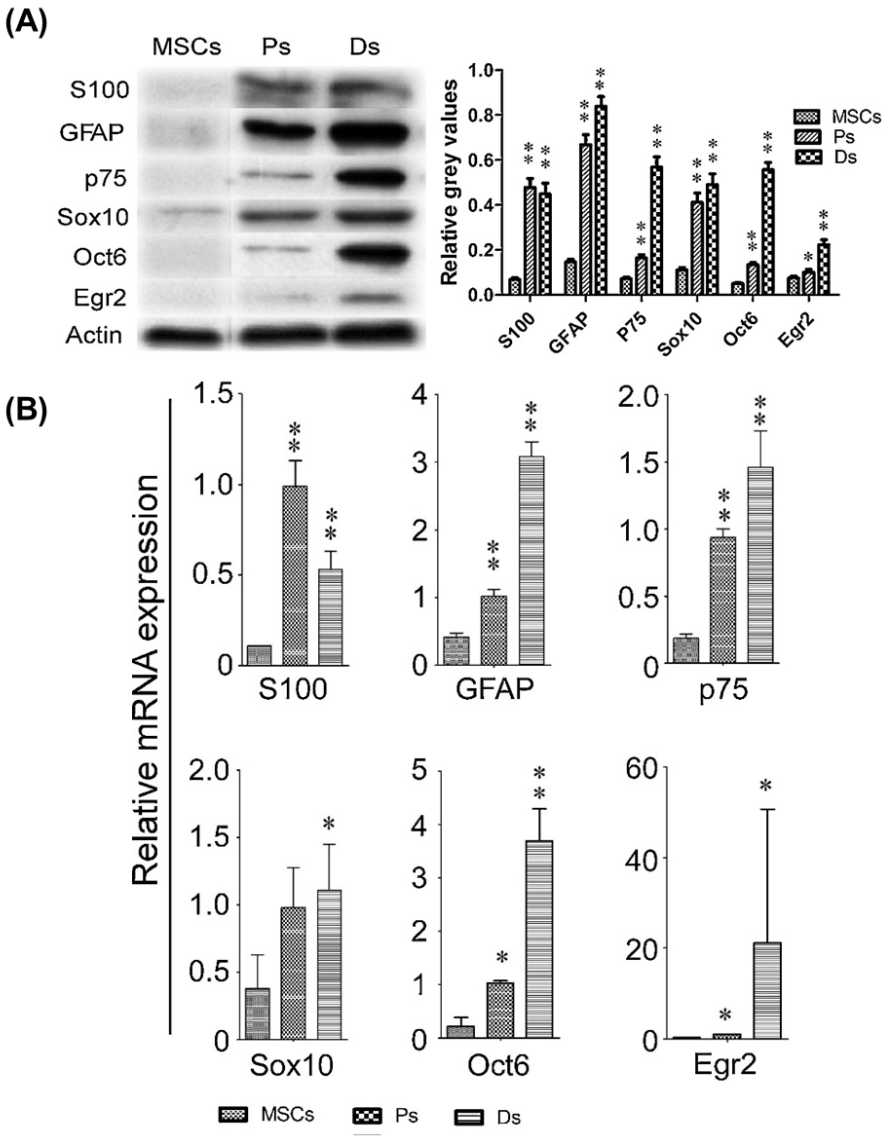

Figure 3. (A) Detection of the protein levels of Schwann cell markers in MSCs. **(B)** mRNA expression of Schwann cell markers in MSCs. The cells cultured with distal segment (Ds) extracts had significantly higher expression of glial fibrillary acidic protein (GFAP), Sox10, Oct6, and early growth response 2(Egr2) than that of cells cultured with proximal segment

(Ps) extracts and the untreated cells. Reproduced with permission.[178] Copyright 2013, Elsevier.

As another strategy, dissociation of neurospheres generated from adipose-derived mesenchymal stem cells[181] or tonsil-derived mesenchymal stem cells (T-MSCs)[182] by culturing with different factors (i.e. bFGF, EGF, and B27 supplement), and subsequent mitogen withdrawal was used to obtain SC-like phenotypes. The SC-like transdifferentiated stem cells promoted myelination and regeneration of damaged nerves in an *in vivo* mouse model of sciatic nerve injury. [182] They also hypothesized that the stem cell differentiation is mediated via stimulating the MEK-ERK1/2 and PI3K-AKT pathways.

Specific receptors, inhibitors or signaling pathways have also been targeted for stem cell differentiation. For instance, considering that tropomyosin receptor kinase A (TrkA) is absent in un-differentiated BMSCs but is expressed in neurally differentiated BMSCs, Zheng et al.[183] transduced rat BMSCs with recombinant lentiviruses to obtain TrkA-overexpressing BMSCs, which were then transdifferentiated into SCs upon transplant into rat sciatic nerve defects via acellular nerve grafts. They expressed the mechanism of differentiation based on NGF binding to TrkA, which leads to receptor dimerization and kinase activation. After phosphorylation, tyrosine Y490 creates sites for adaptor protein Shc while tyrosine Y785 creates sites for phospholipase C-γ1 (PLC-γ1). These receptors are then further involved in intracellular signaling cascades, including the Ras/Erk protein kinase pathway, the phosphatidylionsital-3-OH kinase (PI3K)/Akt kinase pathway and PLC-γ1 pathway regulating NGF/TrkA dependent cell survival and differentiation.

As another approach, combined sequential treatment with inhibitors of the TGF-β and GSK-3 signaling pathways, and with neuregulin-1 to differentiate human pluripotent stem cells (hPSCs) into self-renewing Schwann cell precursors (SCPs) and further into immature Schwann cells was used. [184] Their results indicated that NRG1-ErbB signaling, which leads to the activation of complex intracellular signaling pathways, such as MAPK,

phosphatidylinositol 3-kinase, or PLCg, and subsequent gene expression in SCs, is a key directional cue to drive SC fate specification of a plastic intermediate state of hPSC differentiation and that it depends on the function of SOX10. In a very recent study, Bierlein De la Rosa et al. [187] transdifferentiated genetically modified BDNF hypersecreting mesenchymal stem cells into SC-like phenotypes with 30-50% degree of differentiation using both genetic modification and conventional transdifferentiation techniques.

Beside SC-like phenotypes, it was also demonstrated that neural cell-like phenotypes also show potential for effective repair of peripheral nerve defects by providing protection of peripheral nerve injuries, reduced lesions, and improved recovery, though the mechanism of neural-like cells in the peripheral nerve is still unclear. Therefore, adult stem cells (BMSCs or ADSCs) were also differentiated into neural cell (NC)-like phenotypes possessing characteristics of neurons using chemical stimulation based on EGF, bFGF, Neurobasal medium supplemented with N2 and B27, retinoic acid and sonic hedgehog on 2D tissue culture plates[24] or inhibition of activin/nodal/TGF-β and BMP pathways using SB431542 and dorsomorphin, respectively.[26] These studies demonstrated significant immunolabeling with NC markers, such as NeuN, MAP2, Nestin, anti-β-III tubulin etc., mRNA expression of neuron-specific transcription factors Sox1 and Pax6 and mature neuronal marker NF200.

In vitro Co-culture

The use of dynamic *in vitro* co-culture models, simulating the *in vivo* conditions and synergistic interactions, is another strategy for stem cell differentiation (Table 3).

Table 3. Summary of direct transdifferentiation using co-culture approach.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
2D tissue culture plates	Bone marrow-derived mesenchymal stem cells (BMSCs)	Co-cultured BMSCs with SCs using a Millicell system	More than 75% of the BMSCs were SCs marker positive during co-culture although they were observed to loss typical SC-like morphology and revert back to their native appearance after 3 days of co-culture.	Zhou et al.[188]
2D tissue culture plates	Adipose tissue-derived stem cells (ADSCs)	Either chemical induction or co-culture with SCs in transwell culture dishes	Both differentiation method showed similar degree of differentiation confirmed by the detection of S100, nestin and GFAP markers/genes, suggesting that co-culturing ADSCs and SCs may be a simple, effective and practical way for ADSCs transdifferentiation.	Liao et al.[189]
2D tissue culture plates	Neural stem cells (NSCs)	Co-culturing SCs with NSCs	High cell survival rates, enhanced secretion of BDNF and GDNF and expression of Map2 in the co-culture.	Yu et al.[190]

Zhou et al.[188] co-cultured bone marrow stromal cells (BMSCs) with Schwann cells (SCs) using a Millicell system that allows cell growth in the same culture medium without direct

contact. Their results showed that more than 75% of the BMSCs in the indirect co‐culture model were GFAP‐ and S‐100‐positive during co‐culture, indicating positive transdifferentiation into SCs, although they were observed to lose typical SC‐like morphology and revert back to their native appearance after 3 days of co-culture. However, they were not able to explain the cellular mechanisms behind the transdifferentiation. In another study, Liao et al.[189] used ADSCs to differentiate them into SCs either through chemical induction or co-culture with SCs in transwell culture dishes for comparison of these two methods. Their immunostaining and RT-PCR results indicated that both differentiation methods showed similar degrees of differentiation confirmed by the detection of S100, nestin and GFAP markers/genes, suggesting that co-culturing ADSCs and SCs may be a practical approach for neural transdifferentiation of ADSCs. A similar co-culturing strategy was used for neural stem cells (NSCs). Yu et al.[190] reported that co-culturing Schwann cells (SCs) with NSCs promoted differentiation of NSCs into neurons. This strategy, resulting in high cell survival rates, enhanced secretion of BDNF and GDNF and expression of Map2 in the co-culture, is proposed as a new promising approach (Figure 4).

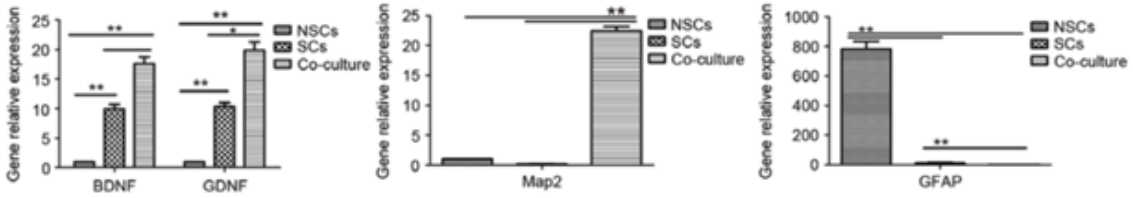

Figure 4. Gene expression for BDNF, GDNF, Map2 and GFAP upon co-culture. Reproduced with permission.[190] Copyright 2017, Spandidos Publications.

***In vitro* Transdifferentiation on 2D Plates Followed by Conduit Transplantation**

Following the *in vitro* transdifferentiation protocols described above (chemical stimulation or *in vitro* co-culture), the differentiated cells were transplanted into animals using NGCs made

from various materials, mostly acellular nerve/muscle grafts, biomaterials or synthetic polymers (Table 4).

Table 4. Summary of direct transdifferentiation using chemical stimuli, conditioned media or co-culture approach followed by transplantation via nerve guidance conduits.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
Acellular muscle stuffed vein	Bone marrow-derived mesenchymal stem cells (BMSCs)	Transdifferentiation via chemical induction on 2D tissue culture plates	35–75% of the cells expressed neural markers after differentiation. Seeded cells produced a new matrix following the degradation of acellular muscle tissue. The vein was intact and no inflammatory reactions were observed.	Hassan et al.[191]
Acellular nerve xenograft	Sprague Dawley (SD) rat hair follicle neural crest stem cells	Obtained neurons and SCs through differentiation of SD rat hair follicle neural crest stem cells with sonic hedgehog/retinoic acid and neuregulin1, respectively	Synergetic effect enhanced regeneration of axons and long-term implanted cell survival upon transplantation.	Lin et al.[192]
Acellular nerve grafts	Adipose tissue-derived stem cells (ADSCs)	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Acellular nerve grafts with differentiated cells showed superior sciatic nerve functional index, wet weight of gastrocnemius muscle, neural electrophysiology,	Gao et al.[193]

			and regenerated myelinated nerve fibers compared to other control groups, but statistically similar to autogenous nerve grafts.	
Acellular nerve graft	ADSCs or BMSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Implantation of differentiated BMSC or ADSC via acellular nerve graft promoted sciatic nerve regeneration and functional recovery in rats, which was comparable to autografting with SCs.	Wang et al.[194]
3D collagen conduit	Human dental pulp stem cells (hDPSCs)	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Promoted and guided neurite outgrowth of DRGs in an aligned tissue-engineered 3D collagen conduit *in vitro.*	Martens et al.[195]
Silicone tube with collagen gels	ADSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	*In vivo* results suggested that both differentiated and undifferentiated cells had potential to enhance axonal regeneration and myelination.	Orbay et al.[57]
Collagen nerve conduits	BMSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Differentiated MSCs in collagen nerve conduits resulted in axonal regeneration equivalent to that of SCs but less effective than the nerve autograft.	Ladak et al.[18]
Microstructured collagen nerve guide (Perimaix)	BMSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture	The pre-differentiated BMSCs seeded in Perimaix nerve guide enhanced axonal	Boecker et al.[196]

		plates	regeneration and myelination but did not significantly improve functional recovery.	
Commercial collagen conduits (NeuraGen® nerve guides)	Schwann cells (SC), SC-like differentiated bone marrow-derived mesenchymal stem cells (dMSC) or SC-like differentiated adipose-derived stem cells (dASC)	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Primary SCs showed significant improvement in distal stump sprouting. No difference in proximal regeneration. Conduits with dMSC and dASC showed diffuse sprouting pattern. Conduits with SCs showed enhanced cone pattern and a typical sprouting along the conduits walls.	di Summa et al.[197]
Genipin crosslinked gelatin annexed with tricalcium phosphate (TCP) ceramic particles	ADSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Promoted proliferation and neuronal differentiation of ADSCs. Conduits with differentiated ADSCs showed morphology and distribution patterns of nerve fibers similar to the autografts.	Liu et al.[198]
Gelatin based matrigel implants	ADSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Good neural differentiation of ADSCs with enhanced BDNF secretion. The transplanted cells via matrigel induced nerve fiber growth.	Lopatina et al.[44]
Fibrin nerve conduits	Schwann cells (SC), SC-like differentiated bone marrow-derived mesenchymal stem cells(dMSC) or	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Fibrin conduit with SCs enhanced axonal regeneration compared to empty fibrin conduit. Both dASCs and dMSCs enhanced regeneration	di summa et al.[199]

	SC-like differentiated adipose-derived stem cells (dASC)		distance.	
Fibrin glue scaffold and collagen tubulization	Porcine skin-derived mesenchymal stem cells (pSMSCs)	Transdifferentiation into neurons via chemical induction on 2D tissue culture plates	The neuron-like cell morphology and characteristic detected at 6h and 24h of induction disappeared as at 48 and 72 h. Transplanted cells showed remarkable *in vivo* nerve regeneration demonstrating histologically complete nerve bundles with highly detected S-100 and p75 markers compared to non-cell grafted control fibers.	Park et al.[200]
Poly (lactic-coglycolic acid) (PLGA, 85:15)	Peripheral blood-derived mesenchymal stem cells (PBMSCs)	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	PLGA conduits with induced PBMSCs provided axonal regeneration and remyelination with expressing myelin specific markers and enhanced nerve conduction recovery, and restoration of motor function, and attenuated myoatrophy and neuromuscular junction degeneration in the target muscle.	Pan et al.[201]
Thin poly (ε-caprolactone) and poly (D,L-lactic acid) based, hydrophobic film scaffolds	ADSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Differentiated ADSC expressed S100 and GFAP markers with high attachment and proliferation rate on the films. They showed functional activity by triggering	Tse et al.[202]

			neurite outgrowth of dorsal root ganglia neurons.	
Poly-3-hydroxybutyrate (PHB) strips with fibrin glue matrix	ADSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	PHB strips seeded with SCs or transdifferentiated ADSCs showed significantly better functional ability than the control groups.	Schaakxs et al.[203]
Poly(DL-lactide-e-caprolactone) (PLC) based commercial membranes (Vivosorbs)	Human umbilicalcord matrix MSCs (HMSCs)	Neuroglial-like differentiation via chemical induction on 2D tissue culture plates	The animals *in vivo* transplanted with either undifferentiated or differentiated HMSCs showed enhanced recovery of motor and sensory function accompanied by an increase in myelin sheath.	Gaertner et al.[204]
Conduit composed of copolymer of 75% L-lactic acid and 25% e-caprolactone filled with atelocollagen (70 wt.% type I and 30 wt.% type III collagen)	Monkey MSCs	Transdifferentiation into SCs via chemical induction on 2D tissue culture plates	Auto-cell transplantation therapy using transdifferentiated MSCs accelerated axonal regeneration and functional recovery in injured nerves.	Wakao et al.[205]
PLA nerve conduits	ASCs or DPSCs	Transdifferentiation into SCs via co-culturing SCs with adipose-derived adult stem cells (ASCs) or dental pulp stem cells (DPSCs)	Synergistic effect of cells provided enhanced secretion of neurotrophic factors *in vitro*. The *in vivo* studies indicated that PLA conduits with co-cultured SCs and ASCs provided improved functional recovery.	Dai et al.[206]
Silk fibroin (SF)/collagen based tissue-engineered nerve conduit	ADSCs	Transdifferentiation into SCs via co-culturing SCs with ADSCs	The transplanted conduits provided accelerated nerve regeneration in 1-cm long sciatic nerve	Xu et al.[207]

			defects in rats.	

Acellular muscle grafts, mimicking the natural nerve structure and elementary nerve ECM, can be obtained from muscles by using chemical extraction procedures. Following transdifferentiation into SCs via regularly used chemical procedures, the transplanted SC-like cells via acellular grafts provided improved PN regeneration. [17, 191] Similarly, a*cellular nerve grafts* obtained from sciatic nerves, were used for stem cell transplantation of different sources (BMSCs or ADSCs) upon transdifferentiation.[193, 194] The acellular grafts with differentiated cells promoted sciatic nerve regeneration, myelination and functional recovery including sciatic nerve functional index (SFI), wet weight of gastrocnemius muscle, neural electrophysiology (NEP), superior to other control groups, but statistically similar to autogenous nerve grafts. In a different study, Lin et al.[192] obtained neurons and SCs through differentiation of Sprague Dawley (SD) rat hair follicle neural crest stem cells with sonic hedgehog/retinoic acid and neuregulin1, respectively. Then, they used acellular nerve xenografts seeded with both neurons and SCs to bridge long-distance gaps in rat sciatic

nerves. They reported that the synergetic effect enhanced regeneration of axons and long-term cell survival upon transplantation.

Biopolymers (Collagen/Gelatin/Chitosan/Fibrin) are considered promising substitutes of autologous nerve grafts. Tissue-engineered 3D collagen conduits or collagen gels/scaffolds filling tubes made of different materials (i.e. silicone) were used with SC-like differentiated stem cells of various sources (human dental pulp stem cells (hDPSCs), BMSCs and ADSCs) in order to facilitate PN regeneration.[18, 57, 195] In general, stem cells transdifferentiated to SC-like cells in collagen nerve conduits showed a greater or equivalent axonal regeneration to that of obtained by undifferentiated stem cells or SCs; however, the nerve autograft still remains the most effective conduit for supporting nerve regeneration.[18, 57] Collagen also serves as the base material of commercially available conduits such as microstructured collagen nerve guide (Perimaix) or collagen based NeuraGen® nerve guides. The SC-like differentiated MSCs or ADSCs were used with Perimaix and NeuraGen® nerve conduits in recent studies and showed high affinity of regenerative cells accompanied with promising axonal regeneration and myelination; however, did not enable significant functional recovery and further modifications were suggested for future tissue-engineering applications (Figure 5).[196, 197]

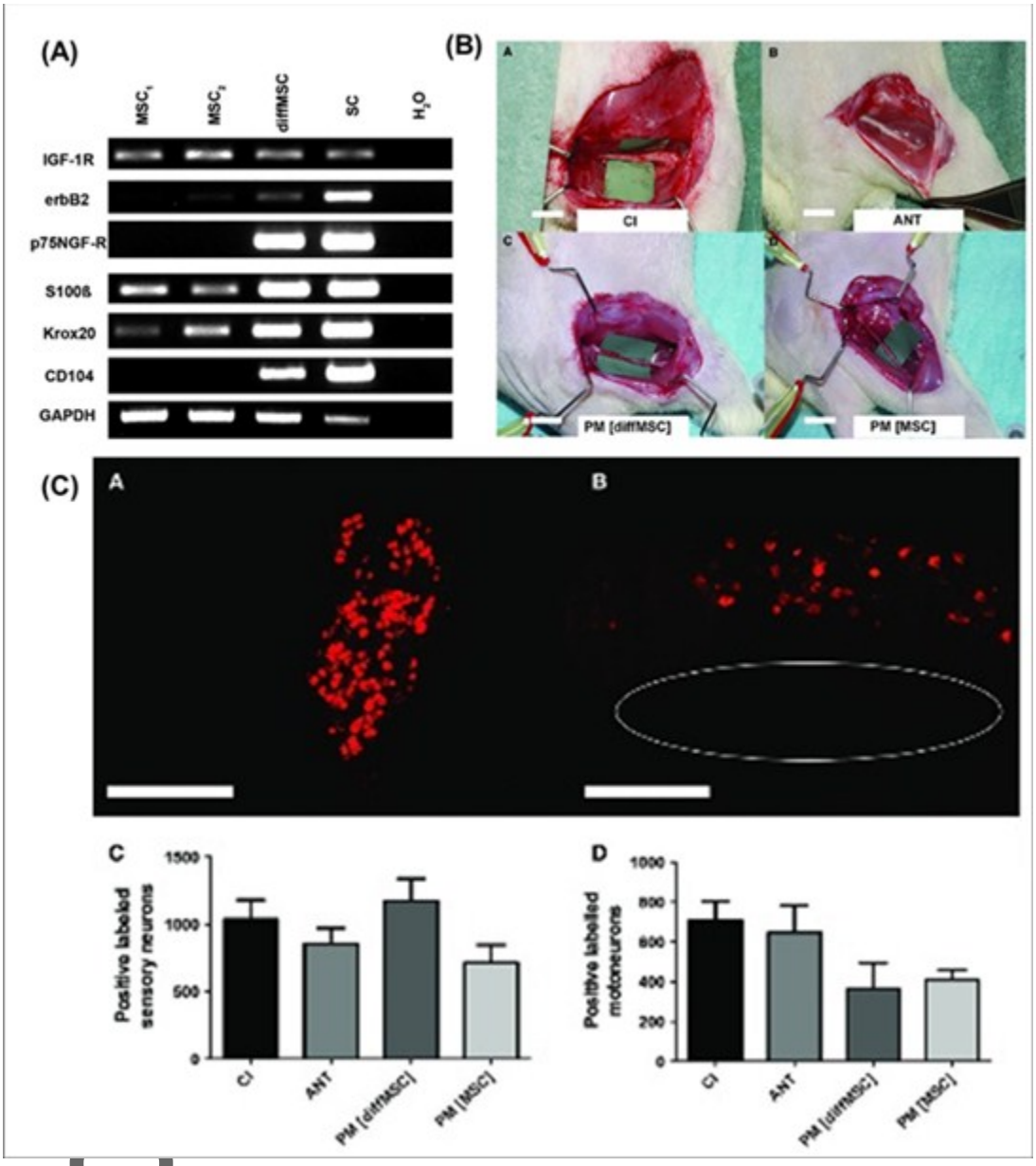

Figure 5. (A) Expression of SC markers in diffMSCs and MSCs. At 3 weeks after cultivation in differentiation medium, diffMSCs expressed the typical SC markers to a similar extent as normal SCs. The marker human epidermal growth factor receptor 2 (erbB2) was less expressed, but still higher than the non-differentiated cells. **(B)** Regenerated sciatic nerves at 12 weeks after implantation. No macroscopic signs of inflammation, hematoma or extensive scar tissue formations were observed. **(C)** Quantification of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) traced sensory and motor neurons at 12 weeks after transplantation. Quantification of positively labelled somatosensory neurons demonstrated similar numbers of regenerated sensory fibres between all experimental

groups. Quantification of the lumbar spinal cord for positively labelled motor neurons revealed a similar pattern as for the sensory fibres; no significant differences in motor neuron counts could be detected between the experimental groups. The functional recovery never reached pre-operative values, but demonstrated enhancement with respect to controls. Reproduced with permission.[196] Copyright 2016, John Wiley & Sons.

Another important biopolymer, gelatin, a derivative of collagen, is also widely used to produce conduits. A biodegradable nerve conduit containing genipin crosslinked gelatin annexed with tricalcium phosphate (TCP) ceramic particles was used with neuron-like differentiated ADSCs[198] while gelatin based matrigel implants[44] were used with ADSCs differentiated into neural lineages. Both studies demonstrated that gelatin based conduits promote cell proliferation and differentiation and enhance nerve fiber growth.

Other biomaterials, such as chitosan and fibrin, are also used to produce conduits. For instance, Li et al.[208] investigated the effect of silanization on porous chitosan conduits in terms of cellular properties and differentiation. Fibrin nerve conduits seeded with various cell types (primary Schwann cells and adult stem cells differentiated to a Schwann cell-like phenotype) were also tested for repair of sciatic nerve injury revealing enhanced regeneration potential of differentiated ADSCs in fibrin conduits.[199] The combined use of different biomaterials for conduit production was also demonstrated. Park et al.[200] used fibrin glue scaffolds and collagen tubulization with neuron-like cell differentiated porcine skin-derived mesenchymal stem cells (pSMSCs). Although the neuron-like characteristics disappeared as the induction time extended to 48 and 72 h, the transplanted cells showed remarkable *in vivo* nerve regeneration with high immunolabelling for S-100 protein and $p75^{NTR}$ in regenerated nerve fibers indicating SC-like differentiation.

Synthetic Polymers (PLLA/PLGA/PCL/PHB) have also been proposed for alternative artificial nerve conduits. Pan et al.[201] prepared a conduit with poly (lactic-coglycolic acid) (PLGA, 85:15) and used peripheral blood-derived mesenchymal stem cells (PBMSCs) that

were induced into SC-like cells for transplantation into crushed rat sciatic nerves. The results indicated that the induced PBMSCs transplanted in PLGA conduits wrapped the injured axons and provided enhanced axonal regeneration and remyelination by expressing myelin specific markers along with the restoration of nerve conduction and motor function. The attenuated myoatrophy and neuromuscular junction degeneration in the target muscle was also observed.

The use of synthetic polymer based conduits make further modifications possible. For instance, the surface of the aligned and aminolyzed poly-Llactide (PLLA) nanofibrous scaffolds were modified with graphene oxide (GO) nanosheets providing rough and hydrophilic surface microstructure that enabled and promoted aligned SCs proliferation, which can potentially be used for stem cell differentiation.[209] On the other hand, the use of fibers and strips is also another approach to provide guided growth as well as supporting the differentiation. Polyhydroxybutyrate (PHB) fibers of a fixed weight threaded through the lumen of a PHB conduit [210] or PHB strips filled with a fibrin glue matrix containing SC-like differentiated adipose-derived stem cells (dASCs) [203] are good examples. These studies suggested that the differentiated cells kept their functionality during the transplantation while the transplanted undifferentiated MSCs showed *in vivo* differentiation into SCs showing significant functional ability (Figure 6).[203, 210]

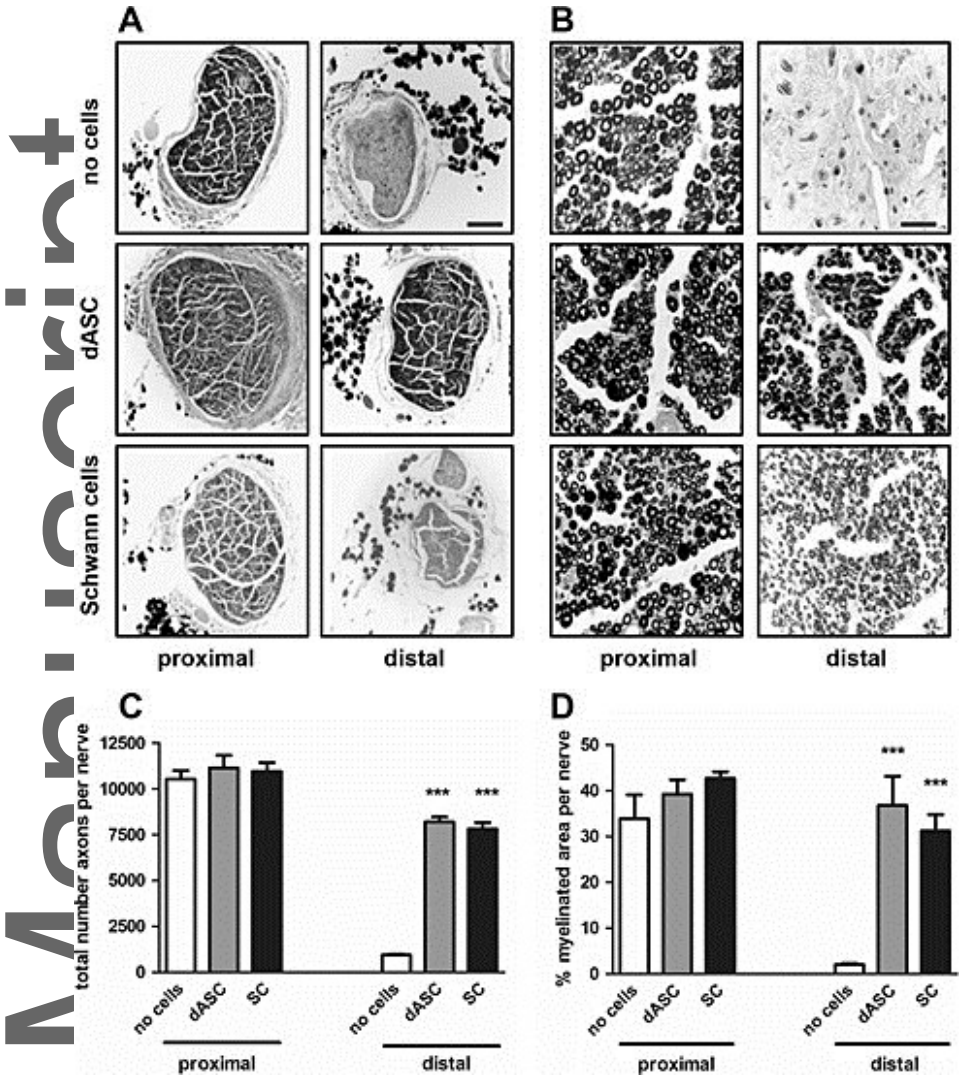

Figure 6. Nerve histology 3 months after surgical implantation of PHB strips. Nerve sections taken from proximal and distal sections **(A)** low **(B)** high magnification. **(C)** Total number of axons/nerve. **(D)** Percent myelinated area/nerve. Morphologically healthy proximal nerves with clear fascicular structures were observed in all groups while the dASC- and SC-treated animals had many regenerated axons in their distal stumps. Significantly higher total numbers of myelinated axons and myelin percentage were observed for both cell type treatment groups in the distal stumps. Reproduced with permission.[203] Copyright 2017, John Wiley & Sons.

Some of these polymers were used in combination to develop conduits and further commercialized to be used in peripheral nerve injuries. Poly (e-caprolactone) and poly (D,L-

lactic acid) based conduits were produced via solvent casting method to create thin, hydrophobic film scaffolds, which can support the differentiation of ADSCs into a Schwann cell-like phenotype and cell transplantation.[202] Another study used Poly(DL-lactide-e-caprolactone) (PLC) based commercial membranes (Vivosorbs) to evaluate the therapeutic value of undifferentiated human umbilical cord matrix MSCs (HMSCs) or neuroglial-like differentiated HMSCs on rat sciatic nerve after axonotmesis injury providing enhanced recovery of motor and sensory function.[204] This study indicated that HMSCs differentiated into neuroglial-like cells, consumed significantly less glucose and produced significantly less lactate compared to native HMSCs that undergo expansion. Based on this observation, they hypothesized that during HMSC differentiation in neuroglial-like cells, the glycolytic process is switched to oxidative metabolism, which could be triggered by several mechanisms, including inhibition of oxidative phosphorylation, dysfunctional Krebs cycle, or inactivation of p53, contribute to the cellular expansion and differentiation. In some cases, synthetic polymers were used in combination with natural biopolymers in order to enhance the regeneration capacity. In one study, a conduit, composed of copolymer of 75% L-lactic acid and 25% e-caprolactone filled with atelocollagen (70 wt.% type I and 30 wt.% type III collagen), was used with SC-like differentiated monkey MSCs in order to observe the regeneration performance for 1 year.[205]

Alternatively, the stem cells, differentiated via *in vitro* co-culture approach, can further be combined with NGCs of synthetic or biopolymers and transplanted into animals to enhance nerve regeneration. Based on this, some studies used adipose-derived adult stem cells (ASCs) or dental pulp stem cells (DPSCs) co-cultured with SCs in PLA conduits or silk fibroin (SF)/collagen based tissue-engineered nerve conduits to treat sciatic nerve defects in rats.[206, 207] This approach provided enhanced nerve regeneration and functional recovery through the synergistic effect of cells providing enhanced secretion of neurotrophic factors.

Scaffolds Materials

In most studies, stem cells were first transdifferentiated *in vitro* into desired cell lineages on two-dimensional (2D) tissue culture plates and then these cells seeded in nerve guidance conduits (NGCs) made from different materials and possessing different physical cues and implanted into the animals. On the other hand, the *in vitro* differentiation of stem cells directly in the NGCs before the transplantation is another strategy saving time and effort (Table 5).

Table 5. Summary of transdifferentiation directly on conduits/scaffolds using chemical stimuli, conditioned media or co-culture approach followed by transplantation.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
Gelatin-based 3D conduits with three different microstructural (nanofibrous, macroporous and ladder-like)	Bone marrow-derived mesenchymal stem cells (BMSCs)	Transdifferentiation into SCs via chemical induction within conduit microstructure	3D ladder-like conduit structure with complex modulus of 0.4x106 Pa and pore size of 150 µm provided the most favorable microenvironment for MSC transdifferentiation leading to 85% immunolabeling of all SC markers and enhanced paracrine activity as well as MSC attachment, proliferation and spreading, creating interconnected cellular networks with large numbers of viable cells.	Uz et al.[122]
PLLA based conduits with different	Neural stem cells (NSCs)	Transdifferentiation into neurons via chemical induction	The nanofibrous structure with higher surface	Zeng et al.[211]

microstructures (nanofibrous, microporous and ladder-like) and microchannels		on conduits	area and lower mechanical strength, resulting from relatively high crystallinity and brittle characteristics, induced the neuronal differentiation of NSCs compared to other structures.	
Polycaprolactone (PCL) electrospun fibers	BMSCs	Transdifferentiation into SCs via chemical induction on conduits	Alignment, diameter, and surface properties of the PCL electrospun fibers promoted the differentiation of BMSCs into SCs, the secretion of neurotrophins and dictated the morphology and alignment of the derived cells.	Xue et al.[123]
Micropatterned PLA and PS films	BMSCs	Transdifferentiation into SCs via chemical induction on conduits	Presence of patterns and type of polymers did not influence the degree of differentiation while the patterns significantly affected the alignment and elongation of differentiated MSCs.	Sharma et al.[19]
PHVB and PEO based electrospun 3D oriented nanofiber scaffolds	Adipose tissue-derived stem cells (ADSCs)	Neuronal transdifferentiation through the temporally sequential use of miR-218 and Fibroblast Growth Factor 2	Demonstrated the neuronal differentiation of ADSCs.	Hu et al.[25]
Photopolymerizable PLL-grafted PEGDA	Neural progenitor	Differentiation of NPC into mature	Hydrogels with 2% PLA grafting also	Cai et

hydrogels	cell (NPC)	neurons via chemical induction on conduits	supported differentiation of NPC into mature neurons with extensive neurite formation and astrocytes rather than oligodendrocytes. However, the hydrogels with 0% and 5% of PLLA grafting did not significantly supported NPC differentiation.	al.[212]
Electrospun Poly(L-lysine) modified zein (ZPLL) nanofibrous membranes with different PLL contents	NSCs	Differentiation of NSCs into mature neurons via chemical induction on conduits	At PLL content of 3.57%, cell adhesion and proliferation proved to be the best and most differentiated toward mature neurons with extensive neurite formation and astrocytes rather than oligodendrocytes.	Miao et al.[27]
Neurotrophin-3 (NT-3) loaded poly-(lactic acid-co-glycolic acid) (PLGA) conduits	Co-seeded NSCs and Schwann cells (SCs)	Differentiation of NSCs into neurons via co-culturing with SCs on conduits	Sustained NT-3 release and co-culturing with SCs triggered the neuronal differentiation of NSCs along with the formation of synaptic structures and myelin sheaths.	Xiong et al.[213]
Layer of reduced graphene oxide (rGO) nanosheets on the surface of bioactive three-dimensional (3D) porcine acellular dermal matrix	BMSCs	Neuronal differentiation via neuronal culture medium on conduits	rGO-assembled PADM scaffold promoted MSCs to neuronal cells differentiation with higher protein and gene expression levels.	Guo et al.[96]

(PADM) channels				
Multiwall CNTs (MWNTs) and polydimethylsiloxane (PDMS) based composite sheets	ADSCs	Differentiation into SCs using a mixture of glial growth factors on conduits	Differentiated ADSCs co-cultured with DRGs enhanced neurite outgrowth.	Han et al.[214]
Polydimethylsiloxane (PDMS) scaffold	Stem cells obtained from human exfoliated deciduous teeth (SHEDs)	Differentiation potential of stem cells obtained from human exfoliated deciduous teeth (SHEDs) into a rat Schwann cell (RSC) on a polydimethylsiloxane (PDMS) scaffold in static and dynamic culture *in vitro.*	Both static and dynamic cultures supported the differentiation, whereas the dynamic culture resulted in formation of neurospheres in higher amounts within significantly shorter times, which could be further differentiated into neurons and neuroglial cells upon culturing in RSCs medium.	Su et al.[215]
3D chitosan conduit	SHEDs	Differentiation potential of stem cells obtained from human exfoliated deciduous teeth (SHEDs) into a rat Schwann cell (RSC) on a polydimethylsiloxane (PDMS) scaffold in static and dynamic culture in vitro	Dynamic culture system generated fluid shear stress and enhanced nutrient transfer, promoting the differentiation of SHEDs to neural cells by expressing the neural stem cell marker.	Su et al.[216]
Chitosan fibers	Neuroepithelial stem cells (NEPs)	Differentiation of NEPs into neurons and glia via chemical induction on conduits	NEPs could firmly attach and grow on the chitosan fibers enabling differentiation of NEPs into neurons and glia.	Fang et al.[217]
Fibrin matrix	Ectomesenchymal stem cells (EMSCs) harvested from	Differentiation into SCs via chemical induction on	Fibrin matrix enhanced the EMSCs proliferation,	Chen et al.[218]

	the nasal respiratory mucosa	conduits	expression of myelination-related molecules, synthesis of neurotrophins and differentiation into SC-like phenotype compared to fibronectin based matrix and 2D tissue culture plates.	
Hyaluronan based membranes and fibrin-glue meshes	Neurospheres isolated from skin and adipose tissues	Differentiate in glial/neuron-like cells on hyaluronan based membranes and fibrin-glue meshes via chemical induction	Neurospheres isolated from skin and adipose tissues were able to differentiate in glial/neuron-like cells on hyaluronan based membranes and fibrin-glue meshes upon induction without any chromosomal imbalance.	Gardin et al.[219]

The conduit properties such as structure, composition and elasticity can influence stem cell differentiation [77, 106, 107, 109, 220] [72, 78, 110-115]. The dimension in which cells are cultured is also a crucial factor in determining the differentiation properties of the cells. The current understanding of most biological mechanisms, including differentiation, has been garnered from cells cultured on 2D surfaces. Most studies examining MSC transdifferentiation have relied on 2D tissue culture plates[15, 17, 20, 21]; however, the cells naturally exist in a complex three-dimensional (3D) microenvironment composed of various ECM molecules, mixed cell populations and cell-secreted factors. Therefore, employing a 3D culture model is more relevant to the physiological condition to explore cell-to-cell and cell-to-matrix interactions related to cellular transdifferentiation. However, the current knowledge with respect to the

influences of mechanical and structural properties of 3D scaffolds on the transdifferentiation behavior of MSCs is significantly limited.[116]

Recent studies have demonstrated that matrix stiffness has a significant influence on the transdifferentiation of MSCs determining not only the differentiating cell lineage but also the triggered signaling pathways. The MSCs cultured on 2D substrates with elastic moduli of lower (0.1–1 kPa), intermediate (8–17 kPa) or higher ranges (34 kPa) showed transdifferentiation tendency into neural, myogenic or osteogenic phenotypes, respectively. Based on this, it was previously reported that the highest expression of neural (ENO2), myogenic (MYOG) and osteogenic (Runx2, OC) transcription factors secreted from MSCs cultured on polyethylene glycol-silica (PEG-silica) based nanocomposite 3D gels were obtained for gels with matrix stiffness of 7, 25 and 75 Pa, respectively. [221] Along with the mechanical properties of conduits, their microstructural properties also influence differentiation of stem cells. In a recent study, gelatin-based 3D conduits with ladder-like structure possessing complex modulus of $0.4x10^6$ Pa and pore size of 150 μm provided the most favorable microenvironment (compared to macroporous and nanofibrous structures) for MSC transdifferentiation leading to 85% immunolabeling of all SC markers and enhanced paracrine activity as well as MSC attachment, proliferation and spreading, creating interconnected cellular networks with large numbers of viable cells (Figure 7). [122] This study anticipated that differentiation of MSCs was modulated by blocking of the BMP/Smad signaling pathway, which promotes integrin internalization, enhancing bone morphogenetic protein receptors (BMPR) endocytosis and neuronal gene expression.

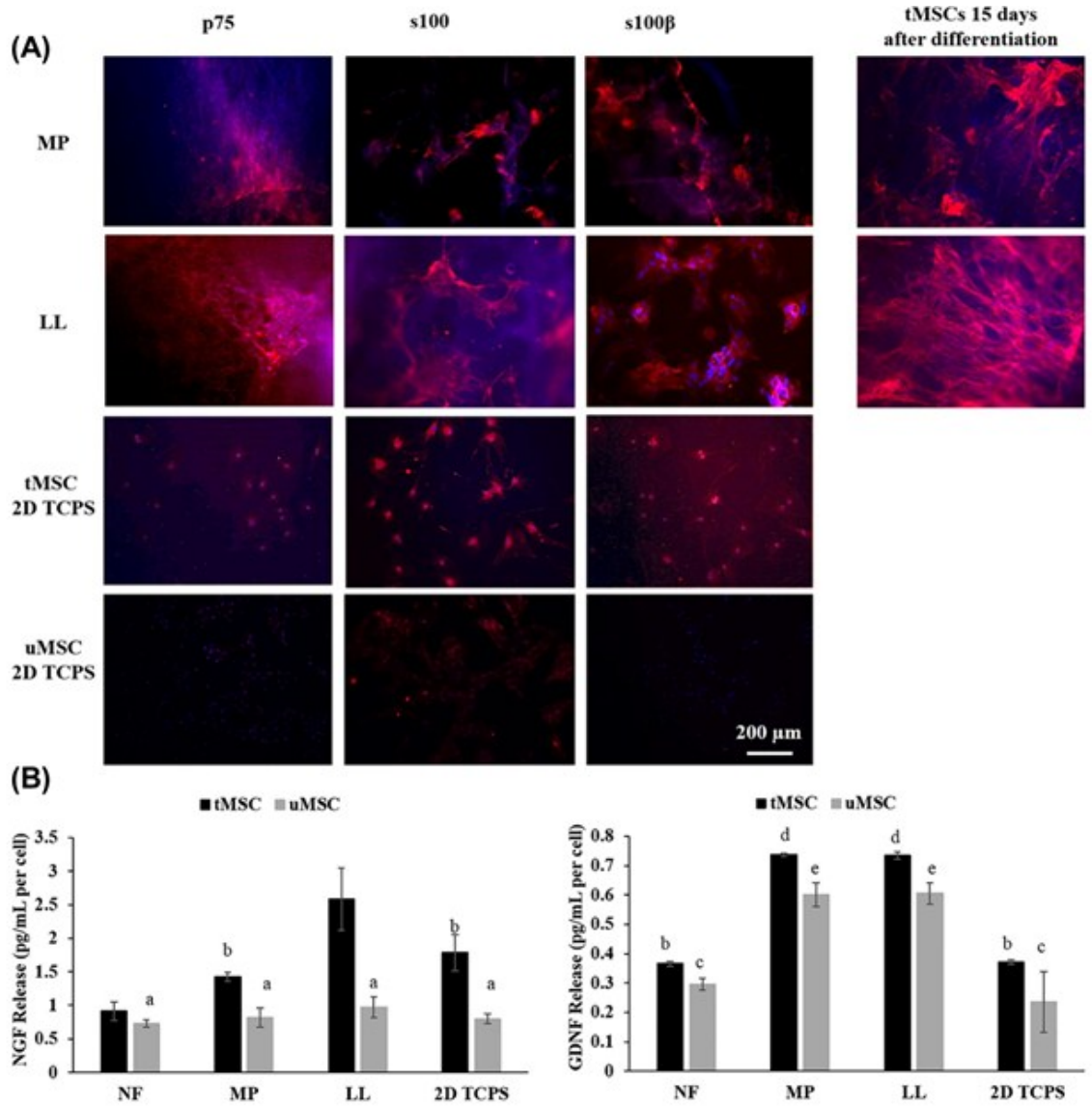

Figure 7. Transdifferentiation of MSCs into SCs within 3D conduits, ladder-like (LL), macroporous (MP) and nanofibrous (NF), and on 2D tissue culture polystyrene (TCPS) plates. **(A)** Immunocytochemical analysis. **(B)** Neurotrophic factor secretion. Reproduced with permission.[122] Copyright 2017, Elsevier.

In addition to the mechanical and microstructural properties, it is also important to use the combination of aligned matrix and optimized differentiation medium properties, which in turn reveals the synergetic influence of extrinsic matrix signaling with intrinsic cell programming for successful differentiation. In a similar study, PLLA based conduits with nanofibrous

structure and microchannels along with higher surface area and lower mechanical strength induced neuronal differentiation of NSCs compared to microporous and ladder-like structures.[211]

In addition to the microchannels, longitudinal fibers or surface micropatterns have also been used to provide guided axonal growth and stem cell differentiation. It was reported that changing the alignment, diameter, and surface properties of electrospun PCL microfibers promoted the differentiation of stem cells into Schwann cells or neurons, the secretion of neurotrophins and dictated the morphology and alignment of the derived cells.[123, 222] These studies showed that the electrospun fibers support stem cell differentiation and alignment. They further demonstrated that fiber-induced cell alignment can effectively activate canonical Wnt signaling in adult NSCs, and further enhance the differentiation induced by biochemical cues mainly by influencing the dynamics of intracellular β-catenin bioavailability. However, in a recent study, it was also reported that the presence of micropatterns or type of polymer films (made from PLA or PS) did not enhance the degree of MSCs transdifferentiation into SCs while significantly affecting the alignment and elongation of differentiated MSCs.[19]

The formulation and composition of the conduit material also determines the performance of the developed conduits and degree of differentiation. Recently, Hu et al.[25] demonstrated neuronal differentiation of adipose-derived mesenchymal stem cells (ASCs) through the temporally sequential use of miR-218 and FGF2 directly on PHVB and PEO based electrospun 3D oriented nanofiber scaffolds. In similar studies with different conduit formulations, it was observed that the change of PLL content had a significant influence on cellular properties, such as adhesion, proliferation and differentiation. Photopolymerizable PLL-grafted PEGDA hydrogels with 2% PLL grafting density[23] and electrospun poly(L-lysine) modified zein (ZPLL) nanofibrous membranes with 3.57% PLL content [27] were reported to increase viability, attachment, proliferation, and further support differentiation of

neural progenitor cell (NPC) and neural stem cells (NSCs) into mature neurons with extensive neurite formation, respectively.

The sustained release property and surface modification of the developed conduits was also used to induce the differentiation of stem cells. In the work of Xiong et al.[213] the sustained NT-3 release from poly-(lactic acid-co-glycolic acid) (PLGA) conduits triggered neuronal differentiation of NSCs along with the formation of synaptic structures and myelin sheaths when co-seeded with Schwann cells (SCs). The surface modification of conduits with carbon based conductive materials (i.e. reduced graphene oxide (rGO) or multiwall CNTs (MWNTs)) is a recent strategy to support stem cell differentiation.[96, 100, 214] It was shown that 3D porcine acellular dermal matrix (PADM) channels modified with assembled layers of rGO nanosheets promoted the differentiation of MSCs into neuronal cells in the presence of neuronal culture medium.[96] In another study, MWNTs and polydimethylsiloxane (PDMS) based composite sheets with superior mechanical strength and electroconductivity supported the differentiation of ASCs into SCs in a mixture of glial growth factors.[214]

Numerous studies using different conduit/scaffolds made from various materials (chitosan fibers, fibrin matrix, hyaluronan-based membranes and fibrin-glue meshes) have been used to differentiate specific stem cell types (ectomesenchymal stem cells (EMSCs) harvested from the nasal respiratory mucosa, neuroepithelial stem cells (NEPs), neurospheres generated from skin and adipose tissues) into glial/neuron-like cells upon chemical stimuli.[217-219] Among these cell types, stem cells from human exfoliated deciduous teeth (SHEDs), an alternative source of adult stem cells, were shown to exhibit a spindle fibroblastic morphology in static culture while they aggregated into neurosphere-like clusters under a dynamic culture.[215, 216] It was shown that both static and dynamic cultures supported the differentiation of SHEDs into a rat Schwann cell (RSC), whereas the dynamic culture resulted in formation of neurospheres in higher amounts within significantly shorter times, which could be further differentiated into neurons and neuroglial cells upon culturing in

RSCs medium. The further use of dynamic culture systems with 3D chitosan conduits generated fluid shear stresses and enhanced nutrient transfer, promoting the differentiation of SHEDs to neural cells.[216]

6. Stem Cell Differentiation via Electrical or Electrical/ Chemical Stimuli

Electrical fields within the ECM have been known for centuries, but their exact nature in terms of time-dependent voltage gradients and its role in developmental processes of human/living organisms have only recently been investigated.[150, 223, 224] These electrical gradients are being related to the spatial variations in ion pumps or leakage across individual cells or across layers of cells. Stem cell differentiation has conventionally been conducted via chemical stimuli, however numerous reports have demonstrated that electrical stimuli have augmented or in some cases circumvented chemical stimuli (Table 6).[90-93, 95]

Table 6. Summary of transdifferentiation using electrical stimuli either alone or in combination with chemical induction.

Conduit/Scaffold Type	Stem Cell Source	Differentiation Method	Outcome	Reference
2D tissue culture plates	Bone marrow-derived mesenchymal stem cells (BMSCs)	Combined chemical and electrical stimuli based differentiation	Electrical stimuli significantly decreased proliferation rate, but increased neuronal differentiation.	Kim et al.[90]
2D tissue culture plates	Human mesenchymal stromal cells (hMSCs)	Combined chemical and electrical stimuli based differentiation	Electrical stimuli enhanced hMSCs into SCs transdifferentiation and enhanced the Schwann cell-like functional behavior and functional activity via the induction	Kim et al.[225]

			of neurotrophic factor release and guided axonal outgrowth *in vivo*.	
Poly(3,4-ethylenedioxythiophene) (PEDOT)–reduced graphene oxide (rGO) hybrid microfiber scaffold	BMSCs	Combined chemical and electrical stimuli based differentiation	Enhanced proliferation and neural Differentiation.	Guo et al.[100]
Graphene-based substrate	Bone marrow-derived human mesenchymal stem cells (hMSCs)	Combined chemical and electrical stimuli based differentiation	Graphene-based substrates support neuronal differentiation of stem cells up-regulating cell through intracellular calcium influx and activated focal adhesion kinase signaling pathway.	Lee et al.[91]
Flexible, inkjet-printed graphene interdigitated electrode (IDE) circuit	BMSCs	Sole electrical stimuli	Differentiation of MSCs into SCs via electrical stimuli on graphene based substrates was as effective as conventionally used chemical stimuli.	Das and Uz et al. [95]
Graphene oxide (GO) nanosheets modified aligned and aminolyzed poly-Llactide (PLLA) nanofibrous scaffolds	Schwann cells	No differentiation	Enabled aligned and promoted SCs proliferation and can potentially be used for stem cell differentiation.	Zhang et al.[209]

For example, differentiation of MSCs to SCs via chemical induction methods using specific compositions of culture media (using expensive growth factors) is well studied, including our own previous work.[16, 17, 122, 226] However, the effect of electrical stimuli either alone or in combination with chemical induction on the differentiation of adult stem cells of different sources into SCs or neurons have recently been investigated.[90-95, 100, 227] The link between electrical stimuli and stem cell differentiation has been at least loosely attributed to the regulation of various signaling pathways such as, FAK and p38[91, 96-100], ion channels and ERK pathway[102-104], MAPK, PI3K, and ROCK,[92, 101] and ROS.[92, 105] Several theories such as differentiation via cell adhesion[228], cell proliferation[229], cell migration[230], and protein generation[231] have been proposed to help explain the connection between electrical stimuli and cellular differentiation. One study (Kim et al.[90]) indicated that intracellular Ca^{2+} signaling that is induced by ELF-EMF is a novel regulatory mechanism that controls neural differentiation of BM-MSCs. They also hypothesized that the signaling pathways related to ferritin has potential influence on neural differentiation. However, further investigation needs to be performed to elucidate the physicochemical underpinnings behind this connection. Nevertheless, there are recent studies investigating the effect of electrical stimuli parameters (such as, voltage, frequency, electrical field strength and so on) along with potential conductive material based conduits/scaffolds targeting the stem cell differentiation and peripheral nerve regeneration. The following section reviews the recent progress made in

stem cell differentiation and neural cell growth via solely electrical or electrical assisted stimulation.

Numerous scaffold materials including those comprised of conductive polymers (e.g., PANI, PEDOT) as well as carbon nanomaterials and even silk have been used to *directly* stimulate cells for neural regeneration purposes. For example, electrical stimulation via polyaniline-based nerve growth conduits has demonstrated increased cellular growth and proliferation than unstimulated cells. Protonated PANI conduits used to produce an electrical field of 10 mV/cm – 2 V/cm was able to alter the cytoskeletal arrangement of hMSCs to produce long filopodial extensions and eventually neural-like cells.[232] A blended PLLA/PANI scaffold was used to electrically stimulate rat nerve cells which resulted in processes with lengths of 24 ± 4 μm compared to 15 ± 3 μm without electrical stimulation.[233] As mentioned previously, graphene-PEDOT hybrid microfibers and inkjet printed graphene micro/nanostructured via a rapid pulse laser technique has been used to differentiate MSCs into neural-like and Schwann-like phenotypes respectively.[95, 100] The former using chemical fibroblast growth factors along with electrical stimuli to induce MSC differentiation and the latter using electrical stimuli only (Figure 8).

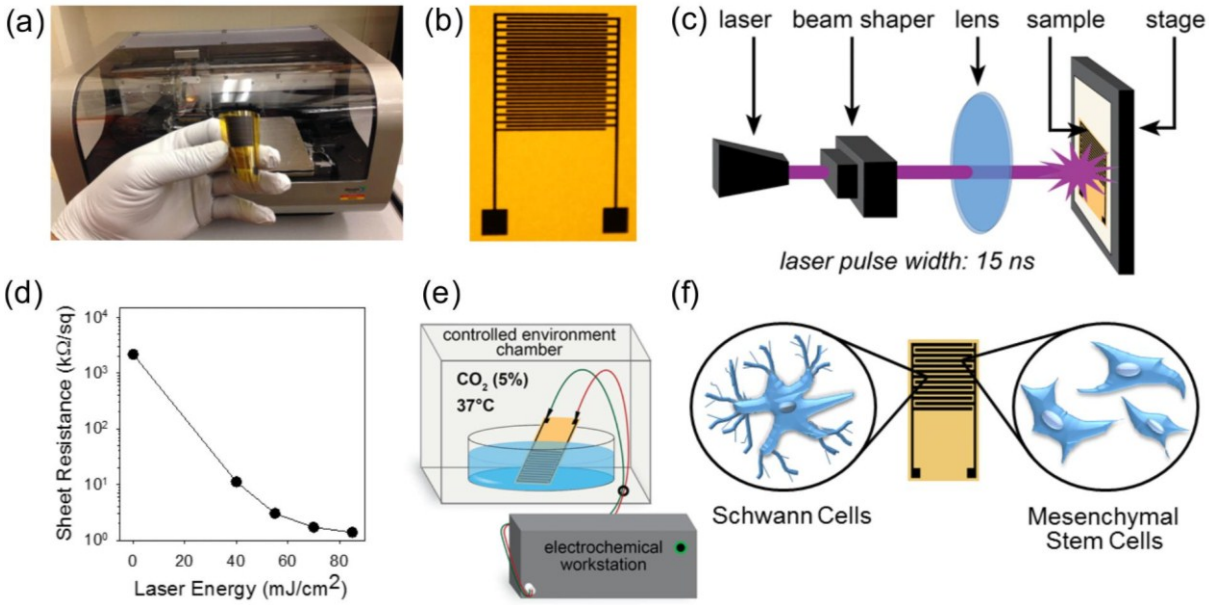

Figure 8. Transdifferentiation of MSCs into SCs on graphene substrates via electrical stimuli. SC-like MSCs were obtained by applying chemical free electrical stimuli of 100 mV at

50 Hz during 10 min for 15 days on laser annealed interdigitated graphene circuits. Reproduced with permission.[95] Copyright 2017, Wiley.

Patterned electronic silk films (derived from *Bombyx mori* silkworm) were capable of enhancing axon outgrowth and alignment via electrical stimulation (120 mV, 1 kHz, for 45 min daily) and surface patterning (grooves with dimensions 3.5 μm width and 500 nm depth) over silk patterns that did not include electrical stimulation or patterning.[234] Other research groups have explored the effects of long-term (i.e., 4 weeks) electrical stimulation in the form of biphasic electric current (BEC) to stimulate axonal regeneration by differentiating hMSCs to into Schwann cells.[235] The BEC stimulation increased the functional activity of Schwann cell and significantly increased axonal outgrowth. Moreover, the concomitance of Schwann cell implants with electrical stimulation (+ 50 mV mm^{-1}dc) has demonstrated a 3.2-fold increase in neurite outgrowth over unstimulated control neurons.[236] Others have demonstrated that 'electro-acupuncture' (i.e., electrical stimulation) at spinal cord injury sites alone, and more significantly with the implantation of MSCs, further lead to partial functional recovery after a spinal cord injury.[94] It is also important to note that such electrical fields have shown to align the migration of neuronal stem/progenitor cells to the cathode and hence neuronal cell regrowth could effectively be directed towards the injury site.[223]

Other researchers have used various *indirect* or non-contact methods to stimulate stem cell differentiation and/or neural cell growth. For example, graphene-PET mixed materials have been used to stimulate neural cells via non-contact electrical field stimulation.[237] Results demonstrate that this applied electrical field promoted new cell-to-cell coupling and strengthened existing cell-to-cell coupling which could facilitate the joining and growth of implanted neural cells to existing cells at a nerve damage cite. Likewise, other research groups have used now direct means to stimulate neural cell regrowth via low intensity pulsed ultrasound (LIPUS).[238, 239] These reports demonstrate that LIPUS might prompt a faster regeneration of the autografting sciatic nerve in the rat model. Others have

demonstrated that extremely low-frequency electromagnetic fields (ELF-EMF) can induce neural differentiation in bone marrow derived MSCs by significantly decreasing the rate of proliferation, which subsequently increases neuronal differentiation.[90] Others have shown that ELF-EMF synergistically increased biological efficacy of neuronal differentiation in bone marrow-derived hMSCs grown on graphene-coated substrates.[91, 92]

These studies showed that neuronal differentiation is achieved by altering global gene expression profile via ELF-EMF, which up-regulates cell adhesion through intracellular calcium influx and integrin mediated focal adhesion kinase signaling pathway that is stimulated by extracellular matrix production. The up-regulation of calcium signaling and phosphorylation of cAMP response element binding (CREB) pathway enhance the neuronal differentiation.[91]

7. Conclusion and Future Perspective

The application of cell-based nerve regeneration therapies has been a promising strategy for the treatment of large PN injuries including for several clinical studies as summarized previously. The transdifferentiation of adult stem cells into SC or NC-like phenotypes via chemical, electrical or synergetic co-culture stimuli or their various combinations that have recently been explored for PN regeneration, have considerable translational potential for cell-based nerve regeneration therapies using nerve guidance conduits with desired features and autologous transplantation. The chemical stimuli based transdifferentiation strategies involves the use of expensive chemicals and growth factors while the synergetic co-culture of different cells (mostly SCs with stem cells) for transdifferentiation that suffers from lack of cell availability and slow *in vitro* growth. Both of the strategies have difficulties in controlling the final fate of the implanted, transdifferentiated cell population since most often the

transdifferentiated cells show tendency to revert back to their original state when the transdifferentiation conditions are removed. Besides these strategies, to date no research has elucidated the strength and duration of the applied electrical field to control the stem cell transdifferentiation process.

A few reports have suggested strategies for transdifferentation of stem cells using combined chemical and electrical stimuli and a very recent study demonstrated the potential of sole electrical stimuli in transdifferentiating the stem cells. Such use of electrical stimuli along with ideal conduit structures may be a promising approach to enable transdifferentiation but also serve to regulate stem cell fate commitment and peripheral nerve regeneration. In addition, the strategy of using electrical stimuli via nerve guidance conduits has also potential to provide direct *in vivo* and in situ transdifferentiation and nerve regeneration upon implantation. Moreover, the spatial control of the electrical field using sophisticated devices or conduits could enable simultaneous and local differentiation of same stem cell sources into different lineages (SCs and neuronal-like phenotypes) and could also impact migration to act synergistically for enhanced PN regeneration. Moreover, nerve growth conduits manufactured with sufficiently conductive, flexible, biocompatible, and microstructured materials for cellular adhesion, growth, differentiation and proliferation still need to be developed to perform *in vivo* nerve regeneration via implantation and differentiation of stem cells. Cellular mechanisms behind stem cell differentiation and nerve regeneration via electrical stimulation should also be elucidated in more detail along with the cellular interactions with the physical cues of conduits, such as microstructural and mechanical properties. Such study directions will pave the way for stem cell based therapies for peripheral nerve regeneration and facilitate their translation to clinical applications.

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The use of adult stem cells holds considerable potential for nerve regeneration applications due to their ability to differentiate into specific cell lineages. This progress report particularly focuses on the recent advances in adult stem cell differentiation strategies including scaffold/conduit materials and electrical /chemical stimulation methods for peripheral nerve regeneration.